Access DB# _____

SEARCH REQUEST FORM

Scientific and Technical Information Center

Requester's Full Name: _____ Examiner # : _____ Date: _____

Art Unit: _____ Phone Number 30 _____ Serial Number: _____

Mail Box and Bldg/Room Location: _____ Results Format Preferred (circle): PAPER DISK E-MAIL

If more than one search is submitted, please prioritize searches in order of need.

****

Please provide a detailed statement of the search topic, and describe as specifically as possible the subject matter to be searched. Include the elected species or structures, keywords, synonyms, acronyms, and registry numbers, and combine with the concept or utility of the invention. Define any terms that may have a special meaning. Give examples or relevant citations, authors, etc, if known. Please attach a copy of the cover sheet, pertinent claims, and abstract.

Title of Invention: _____

Inventors (please provide full names): _____

_____

Earliest Priority Filing Date: _____

For Sequence Searches Only* Please include all pertinent information (parent, child, divisional, or issued patent numbers) along with the appropriate serial number.

Point of Contact:
Alex Weclawiw
Technical Info. Specialist
OWF 12C14 Tel: 308-4491

****

STAFF USE ONLY	Type of Search	Vendors and cost where applicable
Searcher: _____	NA Sequence (#)_____	STN _____
Searcher Phone #: _____	AA Sequence (#)_____	Dialog _____
Searcher Location: _____	Structure (#) _____	Questel/Orbit _____
Date Searcher Picked Up: _____	Bibliographic _____	Dr.Link _____
Date Completed: _____	Litigation _____	Lexis/Nexis_____
Searcher Prep & Review Time: _____	Fulltext _____	Sequence Systems _____
Clerical Prep Time: _____	Patent Family _____	WWW/Internet _____
Online Time: _____	Other _____	Other (specify)_____

PTO-1590 (1-2000)

Bacterial superantigen-induced human lymphocyte responses are nitric oxide dependent and mediated by IL-12 and IFN-gamma.

Sriskandan S, Evans TJ, Cohen J

Department of Infectious Diseases and Bacteriology, Royal Postgraduate Medical School, Hammersmith Hospital, London, United Kingdom.

Bacterial superantigens cause marked proliferation of T cells and release of lymphokines. Nitric oxide, derived from the conversion of L-arginine to L-citrulline, inhibits this activation in murine cells. We have now investigated the roles of IL-12, IFN-gamma, lymphotoxin-alpha, and nitric oxide during superantigen-induced human lymphocyte activation. Lymphocyte activation was determined by measurement of proliferative responses and lymphokine release. Both toxic shock syndrome toxin-1 from Staphylococcus aureus and recombinant streptococcal pyrogenic exotoxin A induced proliferation and production of IFN-gamma, lymphotoxin-alpha, and IL-12 by human mononuclear cells in a time-dependent fashion. The release of IFN-gamma was abrogated by a neutralizing Ab to IL-12, but lymphocyte proliferative responses were unaffected. A neutralizing Ab to IFN-gamma prevented the release of lymphotoxin-alpha, but did not affect proliferation. The neutralization of lymphotoxin-alpha using two different Abs did not affect IFN-gamma release or proliferation. In contrast to previous findings in mice, the arginine analogue, NG-monomethyl-L-arginine, significantly inhibited both proliferation and lymphokine release by superantigen-stimulated human cells. Thus, the release of lymphotoxin-alpha by lymphocytes following superantigen stimulation is dependent upon the presence of IFN-gamma; the IFN-gamma response is in turn under the control of IL-12. There is no evidence that nitric oxide plays an inhibitory role during superantigen-mediated human lymphocyte activation. Indeed, arginine is a prerequisite for such activation.

PMID: 8786301

1: *J Neuroimmunol* 1998 Aug 14;89(1-2):206-12 Related Articles, Books, LinkOut

Vasoactive intestinal peptide inhibits IL-12 and nitric oxide production in murine macrophages.

Xin Z, Sriram S

Vanderbilt University Medical Center, Nashville, TN, USA.

Vasoactive intestinal peptide (VIP) is a naturally occurring neuropeptide widely distributed in the nervous system. In this study, we investigated the effect of VIP on IL-12, TNF alpha and nitric oxide (NO) production in macrophages following activation with lipopolysaccharide (LPS) or superantigens. In vitro studies show that at physiologic concentrations, VIP inhibited IL-12 and NO but not TNF alpha production in macrophages which were stimulated with LPS or superantigens. The inhibitory effect of VIP on IL-12 production appeared to be cAMP mediated since other cAMP inducing agents were also potent in inhibiting IL-12 production. Since IL-12 plays a critical role in T cell function, we suggest that naturally occurring neural hormones can regulate the type and direction of the immune response.

PMID: 9726844

1: *Blood* 1995 Sep 15;86(6):2429-38 Related Articles, Books, LinkOut

Interleukin-12 inhibits murine graft-versus-host disease.

Sykes M, Szot GL, Nguyen PL, Pearson DA

Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston 02129, USA.

Interleukin-12 (IL-12) is a potent immunostimulatory cytokine and an inducer of type-1 T-helper cell activity and of cytotoxic T lymphocyte and natural killer cell function. We report here the paradoxical observation that a single injection of 4,900 IU of recombinant murine IL-12 inhibits acute graft-versus-host disease (GVHD) in a fully major histocompatibility complex (MHC) plus multiple minor antigen-mismatched bone marrow transplantation (BMT) model (A/J-->B10). The protective effect was enhanced by administration of T-cell-depleted host-type BM cells, and complete donor-type lymphohematopoietic reconstitution was observed in most animals. Treatment with a protective course of IL-12 led to increased serum interferon-gamma (IFN-gamma) levels as compared with those for GVHD controls at early time points, when IFN-gamma was produced predominantly by host-type natural killer cells, but led to almost complete inhibition of the later GVHD-associated increase in serum IFN-gamma levels, when IFN-gamma is produced predominantly by CD4+ T cells. Furthermore, IL-12 treatment was associated with marked alterations in the kinetics of donor T-cell expansion. Reductions in donor CD4+ and CD8+ T cells were observed in the spleen on day 4 post-BMT, but a marked increase in donor CD8+ cells was observed on day 7. Unlike broadly immunosuppressive methods for inhibiting GVHD, which are associated with loss of antileukemic effects, IL-12 has the potential to mediate antileukemic effects of its own; therefore, the GVHD-inhibitory effects of IL-12 described here suggest a potential application for this cytokine in clinical BMT.

PMID: 7662991

R850.J6

STIC-ILL

From: Prasad, Sarada
Sent: Tuesday, March 06, 2001 5:33 PM
To: STIC-ILL

Please fetch me the following full text citations for the case # 09,395,038

1. Philosophical transactions of the roayal Society of London B Biological Sciences (Sept. 29, 1997) vol. 352, No. 1359, pages 1311-1315.(cytokines and nitric oxide as effector molecules against parasitic infections.
2. J Immunol. 1994, vol.153, No.11, pages 5200-5209. (Elevated expression of Th1 cytokines and nitric oxide synthase in the lungs of vaccinated mice after challenge infection with schistosoma mansoni.)
3. Cancer immunol, Immunotherpy, 1996 jan, vol.42, No.1, pages 38-46. (Effects of N-methyl arg, an inhibitor of nitric oxide synthesis on IL-2 induced capillary leakage and anti-tumor responses innhealthy and tumor bearing mice.
4. Infection and immunity 1997, vol. 65, No. 4 1307-1312. (Expression of cytokines and inducible nitric oxuide synthase mRNA in the lungs of mice infected with cryptococcus neoformans: effects of interleukin 12).
5. Journal of general virology 1998, vol.79, No. 4, pages 825-830 (Mice lacking inducible niric oxide synthase are more susceptible to herpers simplex virus infection despite enhanced Th1 cells responses)
6. J Neuroimmunology 1996, vol.68, No. 1-2, pages 101-108. (Interferon gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons.)
7. J Experimental medicine 1996, vol.183, No. 4, pages 1447-1459. (The role of interleukin 12 and nitric oxide in the development of spontaneous auroimmune disese in mrl...mice).

thank you,

Sarada Prasad, Ph.D.
Patent Examiner
Art Unit 1646
Room CM1-8B17
Tel: 703-305-1009

The Role of Interleukin 12 and Nitric Oxide in the Development of Spontaneous Autoimmune Disease in MRL/MP-*lpr/lpr* Mice

By Fang-Ping Huang, Gui-Jie Feng, George Lindop,* David I. Stott, and Foo Y. Liew

*From the Department of Immunology and *Pathology, University of Glasgow, Glasgow G11 6NT, United Kingdom*

Summary

MRL/MP-*lpr/lpr* (MRL/*lpr*) mice develop a spontaneous autoimmune disease. Serum from these mice contained significantly higher concentrations of nitrite/nitrate than serum from age-matched control MRL/MP-+/+ (MRL/+), BALB/c or CBA/6J mice. Spleen and peritoneal cells from MRL/*lpr* mice also produced significantly more nitric oxide (NO) than those from the control mice when cultured with interferon (IFN) γ and lipopolysaccharide (LPS) in vitro. It is interesting to note that peritoneal cells from MRL/*lpr* mice also produced markedly higher concentrations of interleukin (IL) 12 than those from MRL/+ or BALB/c mice when cultured with the same stimuli. It is striking that cells from MRL/*lpr* mice produced high concentrations of NO when cultured with IL-12 and LPS, whereas only low or background levels of NO were produced by similarly cultured cells from MRL/+ or BALB/c mice. The enhanced NO synthesis induced by IFN-γ/LPS was substantially inhibited by anti-IL-12 antibody. In addition, IL-12–induced NO production can also be markedly inhibited by anti-IFN-γ antibody, but only weakly inhibited by anti–tumor necrosis factor α antibody. The effect of IL-12 on NO production was dependent on the presence of natural killer and possibly T cells. Serum from MRL/*lpr* mice contained significantly higher concentrations of IL-12 compared with those of MRL/+ or BALB/c control mice. Daily injection of recombinant IL-12 led to increased serum levels of IFN-γ and NO metabolites, and accelerated glomerulonephritis in the young MRL/*lpr* mice (but not in the MRL/+ mice) compared with controls injected with phosphate-buffered saline alone. These data, together with previous finding that NO synthase inhibitors can ameliorate autoimmune disease in MRL/*lpr* mice, suggest that the high capacity of such mice to produce IL-12 and their greater responsiveness to IL-12, leading to the production of high concentrations of NO; are important factors in this spontaneous model of autoimmune disease.

MRL/MP-*lpr/lpr* (MRL/*lpr*)[1] mice develop a spontaneous autoimmune disease and have been used extensively as a model for clinical SLE. The disease is characterized by lymphadenopathy, autoantibody production, and inflammatory manifestations such as nephritis, vasculitis, and arthritis (1, 2). The cause of the disease is likely to be multifactorial, including a single gene mutation (*lpr*) of the *fas* apoptosis gene on mouse chromosome 19 (3, 4) and background genes from the MRL strain (1, 4).

Recent studies show that MRL/*lpr* mice excreted significantly higher concentrations of urinary nitrate/nitrite than age-matched normal C3H mice (5). Furthermore, MRL/*lpr* mice showed markedly reduced proteinuria and minimal glomerular proliferation when treated orally with L-N^G monomethyl arginine (L-NMMA), an inhibitor of nitric oxide synthase (NOS) (5). These data therefore strongly suggest that nitric oxide (NO) is an important mediator of the disease manifestation of MRL/*lpr* mice. However, the mechanism(s) for this exaggerated NO synthesis by MRL/*lpr* mice remains obscure.

NO is a critical mediator of a variety of biological functions, including vascular relaxation, platelet aggregation, neurotransmission, tumoricidal and microbicidal activity, and immunosuppression (6–10). It is also implicated in a range of immunopathologies (11–13). NO is derived from the guanidino nitrogen atom(s) (14) and molecular oxygen (15, 16) in a reaction catalyzed by the enzyme NOS. There are three major isoforms of NOS (17): the neuronal form

[1] *Abbreviations used in this paper:* HRP, horseradish peroxidase; L-NMMA, L-N^G monomethyl arginine; MRL/*lpr*, MRL/MP-*lpr/lpr*; NO, nitric oxide; NOS, nitric oxide synthase.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/04/1447/13 $2.00
Volume 183 April 1996 1447–1459

(nNOS) and the endothelial form (eNOS) produce the amounts of NO required for physiological functions. The cytokine-inducible form (iNOS) is induced by a number of immunological stimuli, such as IFN-γ, TNF-α, and LPS, and catalyzes the high output of NO which can be cytotoxic.

Since the expression and functional activities of IFN-γ and TNF-α in SLE and its animal models are highly variable and controversial (18, 19), we explored the possibility that the exaggerated NO synthesis in MRL/*lpr* mice may be due to enhanced production of other factor(s). We report here that spleen and peritoneal cells from MRL/*lpr* mice produced significantly higher concentrations of IL-12 than those from the control MRL/+, or BALB/c mice when stimulated with LPS and IFN-γ in vitro. IL-12 and LPS synergistically stimulated the spleen and peritoneal cells from MRL/*lpr* mice, but not from the control MRL/+ mice, to produce high levels of NO. Furthermore, young MRL/*lpr* mice developed accelerated glomerulonephritis when injected with rIL-12 compared with control injected with PBS alone. Thus, the enhanced capacity to produce IL-12 and the higher responsiveness to IL-12/LPS to produce NO may play an important role in the pathogenesis of MRL/*lpr* mice.

Materials and Methods

Mice. Female MRL/*lpr* and age- and sex-matched control MRL/MP-+/+, BALB/c, and CBA/6J mice were obtained from Harlan Olac Ltd. (Bicester, UK). Some of the mice were bred in the animal facilities, University of Glasgow, from pairs obtained from Harlan Olac. They were housed in a conventional animal facility.

Cytokines and Reagents. Murine rIFN-γ was a kind gift of Dr. G. Adolf (Bender, Vienna, Austria). Murine rIL-12 and monoclonal (clones C15.6 and C15.1.2) and polyclonal (sheep no. 7) anti-IL-12 antibodies were generously provided by Dr. Stan Wolf (Genetic Institute, Boston, MA). Polyclonal anti-IL-12, anti-IFN-γ, and anti-TNF-α antibodies were raised in rabbits immunized with murine rIL-12, rIFN-γ, or TNF-α, respectively using a standard protocol. Monoclonal anti-CD4 (YTS191) and anti-CD8 (YTS169) were kindly provided by Dr. H. Waldmann (University of Oxford, Oxford, UK). Monoclonal anti-NK antibody (5E6, endotoxin removed) was obtained from PharMingen (San Diego, CA). Monoclonal anti-Thy1.2 (F7D5) was obtained from Olac Ltd. Fresh rabbit serum was used as a source of complement. L-NMMA and D-NMMA were kindly provided by Dr. S. Moncada (Glaxo Wellcome Research Laboratory, Beckenham, UK). LPS (*Salmonella enteritidis*) and Con A were obtained from Sigma (Poole, UK).

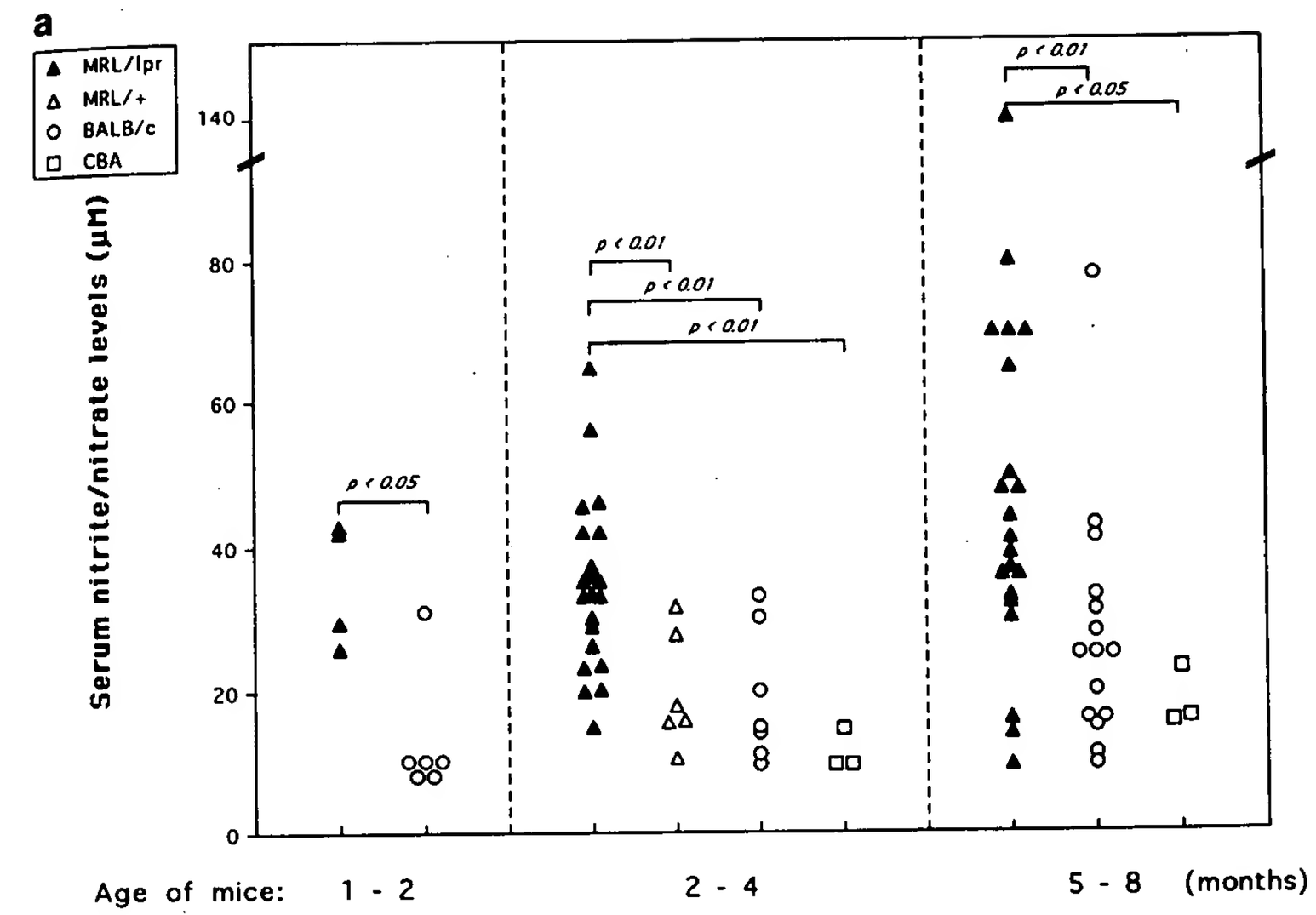
Mouse Peritoneal and Spleen Cell Preparation. Peritoneal cells were collected by injecting 5–7 ml of ice-cold PBS into the peritoneal cavity before harvesting and kept on ice before use. Spleen was then removed and a single cell suspension prepared by gently forcing the spleen through a sterile tea strainer into a petri dish in HBSS (Gibco, Paisley, UK) containing 1% FCS. The cells were then washed in serum-free HBSS and viability determined by trypan blue exclusion.

Cell Depletion. Single cell suspensions (10^7 cells/ml) in PBS were incubated on ice for 30 min with anti-CD4, anti-CD8 (hybridoma culture supernatant, 1:1 dilution), anti-NK (5E6, 5 μg/ml), anti-Thy1.2 (ascites, 1:500 dilution) or anti-Thy1.2, plus anti-NK. After 2 washes with ice-cold PBS, the cells were incubated with or without rabbit complement (1:20) in 96-well culture plates for 45 min at 37°C at 2×10^5 cells/well in 100 μl of complete culture medium (RPMI 1640; Gibco) supplemented with 10% heat-inactivated FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μM 2-mercaptoethanol). Cells were then pelleted by centrifugation of the plate and the supernatant was carefully removed. The incubation was repeated with fresh complement and followed by two washes with warm medium. Samples of the residual cells were phenotyped in parallel tubes by flow cytometry (Becton Dickinson & Co.) using FITC- or PE-conjugated antibodies to CD4, CD8 (Becton Dickinson & Co., Oxford, UK) and CD3 (PharMingen).

Cell Culture. Spleen (2×10^5 viable cells/well) or resident peritoneal cells ($1.5–3 \times 10^5$ cells/well, varied between different experiments) in 200 μl were cultured in full medium in 96-well plates (Nunc, Roskilde, Denmark) at 37°C and 5% CO_2 for up to 6 d. To stimulate for NO synthesis, graded doses of IL-12 and LPS were titrated and optimal doses determined. IFN-γ was used at 50 U/ml unless indicated otherwise. To stimulate for IL-12 production, graded doses of LPS were titrated with 50 U/ml of IFN-γ. In the antibody neutralization experiments, cells were preincubated with specific antibodies to murine IL-12 (sheep no. 7 or rabbit anti-IL-12), IFN-γ, or TNF-α for 30 min at 37°C before the addition of stimulators. Concentrations of antibody used were supraoptimal for neutralizing the amounts of cytokines likely to be produced as determined in preliminary experiments.

Cytokine Assays. IL-12 concentration was determined by an ELISA method using a combination of two rat monoclonal antibodies (C15.1.2 and C15.6, Genetic Institute) to mouse IL-12 (p40 chain) as capture antibodies, and a sheep anti–mouse IL-12 antibody (sheep no. 7) or a rabbit anti–mouse IL-12 (Rab.74.6) as detecting antibody. ELISA in 96-well plates (Immulon 4; Dynatech, Billingshurst, UK) was developed with a biotin-conjugated donkey anti–sheep IgG antibody (Sigma) followed by StrepAvidin-horseradish peroxidase (HRP) or a HRP-conjugated donkey anti–rabbit IgG (SAPU, Carluke, UK) accordingly, TMB HRP substrate (KPL Laboratories, Gaithersburg, MD); optical density was read on a Dynatech MR5000 ELISA reader at 630 nm. Recombinant murine IL-12 (Genetic Institute) was used as standard. Normal donkey serum (2%) was used as blocker. IL-12 production was also determined by Western blot. Peritoneal cells from four 13-wk-old MRL/*lpr* mice were pooled and cultured at 2.5×10^5 cells/ml in 25-cm^2 flasks in the presence or absence of IFN-γ (50 U/ml) and LPS (1 μg/ml). Culture supernatant was harvested at 6, 12, 24, and 48 h, 3-ml samples were immune precipitated with rat monoclonal anti-IL-12 antibodies (clones C15.1.2, and C15.6, both against the p40 chain of IL-12), and the immune complexes were captured by protein A–Sepharose beads. The precipitate was then resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Herts, UK). After blocking with Tris-buffered saline containing 0.1% Tween 20 and 2% BSA, the membrane was incubated sequentially with anti-IL-12 antibody (sheep no. 7), biotin-conjugated donkey anti–sheep IgG, and HRP-conjugated avidin, and protein bands visualized by the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Amersham, Bucks, UK). Recombinant murine IL-12 was run in parallel with the test samples.

The IFN-γ concentration was also determined by ELISA using a rat monoclonal antibody (R46AT) and a rabbit anti–mouse IFN-γ antibody. The assay was developed with an alkaline phos-



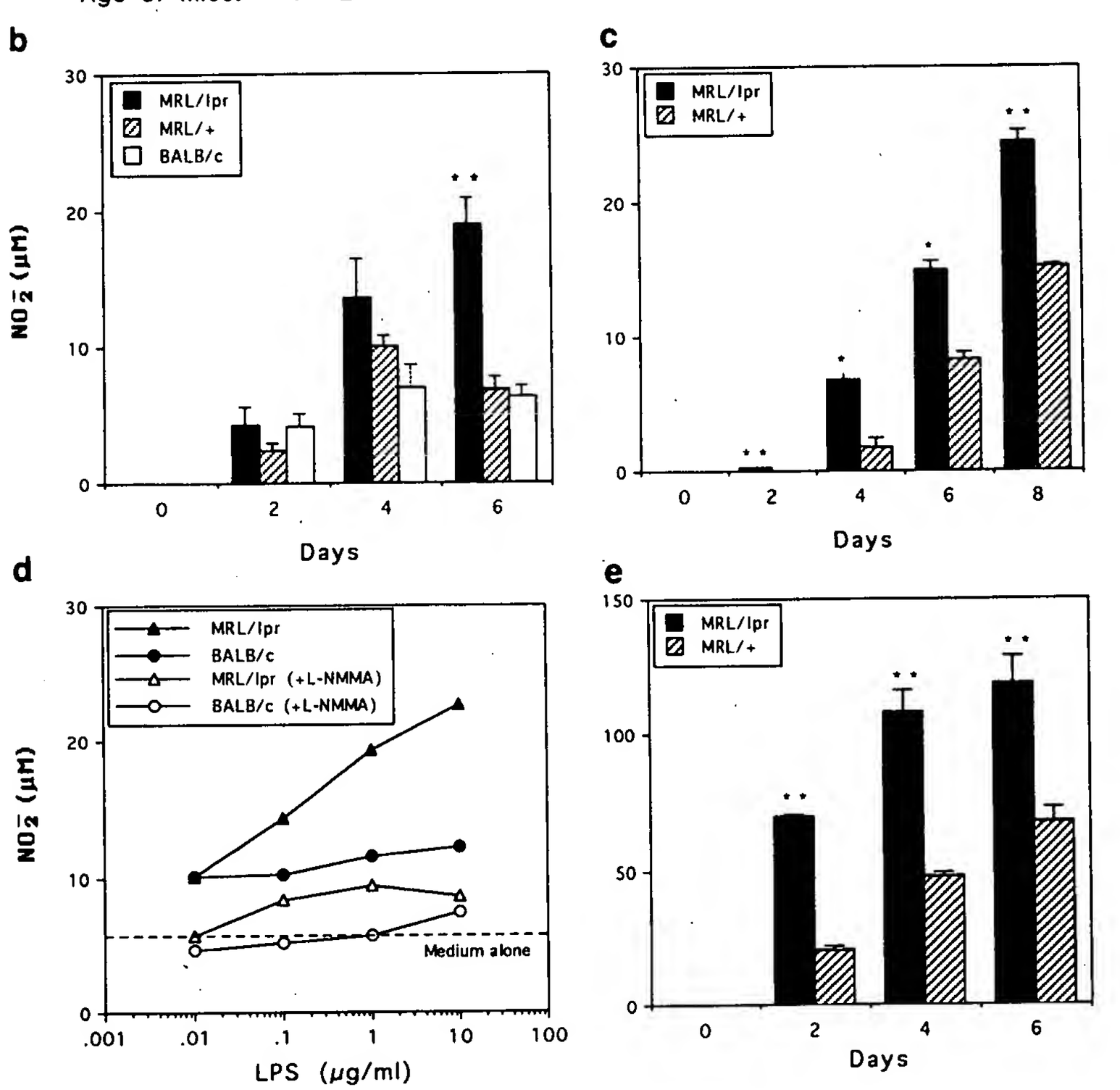


Figure 1. Enhanced NO synthesis in MRL/*lpr* mice in vivo and in vitro. (*a*) Serum nitrite/nitrate levels in MRL/*lpr* lupus strain (n = 47) and three control strains (n = 40) of mice at different ages. Total nitrite and nitrate concentration in serum was determined by the nitrate reductase method which converted nitrate into nitrite before measurement (see Materials and Methods). LPS/IFN-γ–induced NO production by splenic (*b–d*) or peritoneal (*e*) cells from young (6-wk; *b* and *d*) and old (25-wk; *c* and *e*) MRL/*lpr*, MRL/+, or BALB/c mice. Spleen or peritoneal cells from three mice per group were pooled and stimulated in 96-well culture plates with IFN-γ (50 U/ml) and either 1 μg/ml (*b*, *c*, and *e*) or graded doses (*d*) of LPS. In some cultures (*d*) L-NMMA (500 μM) was added. Culture supernatants were collected at daily intervals and nitrite levels were measured by the Greiss method. Data shown are time course (*b*, *c*, and *e*), or (*d*) at day 6 as mean and SD of triplicate cultures. (*P <0.05, **P <0.01). (*d*, *dotted line*) Nitrite level in unstimulated cultures. Consistent results were obtained in more than 10 repeated experiments.

phatase–conjugated goat anti–rabbit IgG antibody (Sigma) followed by *p*-nitrophenyl phosphate. Optical density was read on a Dynatech MR700 ELISA reader at 410 nm. Recombinant murine IFN-γ was used as standard. In some experiments, an ELISPOT assay was also used to enumerate the number of IFN-γ–secreting cells. This was carried out as described previously (20).

Assays for NO Production. Total nitrate and nitrite concentration in serum was determined by the conversion of nitrate into

nitrite as described previously (21). Briefly, serum samples (30 μl) were incubated with an equal volume of reaction buffer containing nicotinamide adenine dinucleotide phosphate (1 mg/ml), flavin adenine dinucleotide (8.3 mg/ml), KH_2PO_4 (0.1 M), and nitrate reductase (0.7 mg/ml; Sigma), added immediately before use. Conversion was carried out at 37°C for 2 h in a 96-well ELISA plate (Immulon 2; Dynatech). Total nitrite content was then measured in a chemiluminescence NO analyzer (Dabisi model 2107; Quantitech Ltd., Milton Keynes, UK) according to the manufacturer's instruction. Nitrate standard was run in parallel with test samples. The assay was performed in triplicate and had a detection limit of 5 μM. Nitrite concentration in culture supernatants was determined in triplicate by the Greiss reaction (22), using $NaNO_2$ as standard with a detection limit of 1 μM.

Detection of Serum Autoantibodies by ELISA. This was carried out as described previously (23) using single (ss) or double (ds) stranded calf thymus DNA (Sigma) as target antigens. Pooled serum from 20-wk-old MRL/*lpr* mice of known high titer of anti-DNA antibodies was used as standard serum. One titration unit was arbitrarily defined as the amount of antibody present in a fixed dilution of the standard serum (1/10,000 for anti-ssDNA and 1/1,000 for anti-dsDNA antibodies).

Renal Histology. Mouse kidney tissues were fixed in formalin and embedded in paraffin; 5-μm sections were stained with periodic acid-Schiff. For histological examination by light microscopy, sections were randomly labeled and examined blind twice by two investigators. The severity of kidney pathology was assessed by the extent of enlargement of glomeruli and mesangial cell proliferation, tuft-to-capsule adhesions, protein casts in tubules, interstitial cellular infiltration, and vasculitis.

Statistical Analysis. Statistical significance (p value) was calculated by the Mann Whitney test (Minitab software program; Minitab Inc., State College, PA).

Results

MRL/lpr Mice Produced Higher Concentrations of NO Metabolites than Normal Mice. Serum from MRL/*lpr*, MRL/+, BALB/c, and CBA mice of various ages were analyzed for NO metabolites by converting nitrate to nitrite and then determining the total nitrite content. Serum from MRL/*lpr* mice consistently contained significantly higher concentrations of nitrate and nitrite than those from age- and sex-matched MRL/+, BALB/c, or CBA/6J mice (Fig. 1 *a*). There was no significant difference between the concentrations of NO metabolites produced by MRL/+, BALB/c, or CBA mice. These results therefore confirm previous findings (5) that MRL/*lpr* mice produce exaggerated levels of NO in vivo.

To analyze the mechanism(s) for the exaggerated production of NO by the MRL/*lpr* mice, spleen (Fig. 1, *b–d*) or peritoneal (Fig. 1 *e*) cells from MRL/*lpr*, MRL/+, or BALB/c mice were cultured with IFN-γ and LPS in vitro for up to 8 d, and the concentrations of nitrite in the culture supernatants determined. Cells from MRL/*lpr* mice consistently produced significantly higher levels of NO than similarly cultured cells from age-matched young (6-wk-old, Fig. 1, *b* and *d*) or old (24-wk-old, Fig. 1, *c* and *e*) MRL/+, or BALB/c mice. The production of NO was LPS dose dependent and was inhibitable by L-NMMA (Fig. 1 *d*). IFN-γ alone or LPS alone induced only a minimum level of NO synthesis by spleen cells (see Fig. 3, *b* and *c*). LPS alone did, however, induce significant levels of NO production by *lpr* peritoneal cells (see Fig. 3 *e*).

Peritoneal and Spleen Cells from MRL/lpr Mice Produce High Concentrations of IL-12. We next investigated the produc-

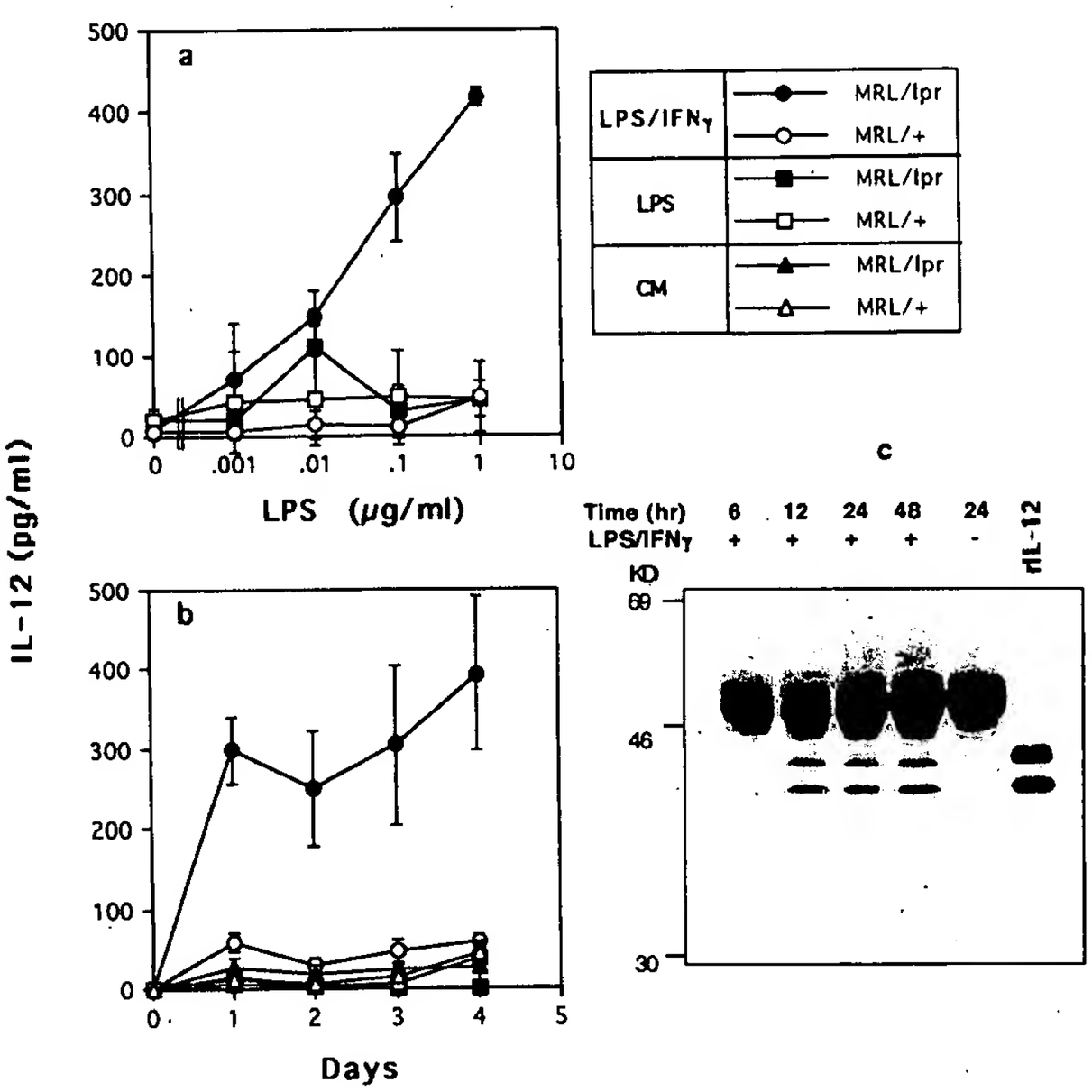
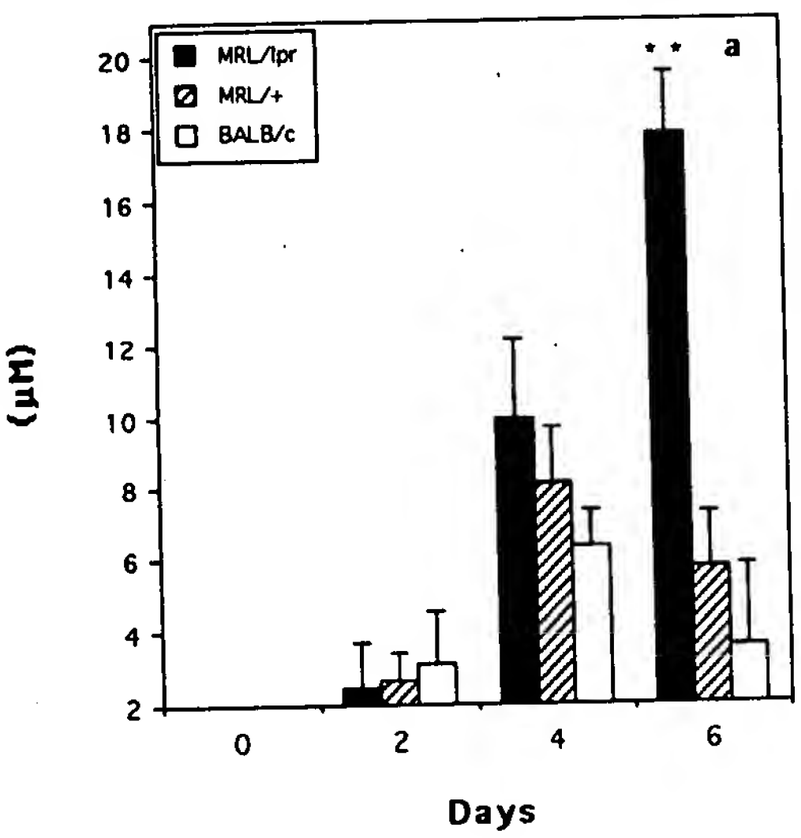
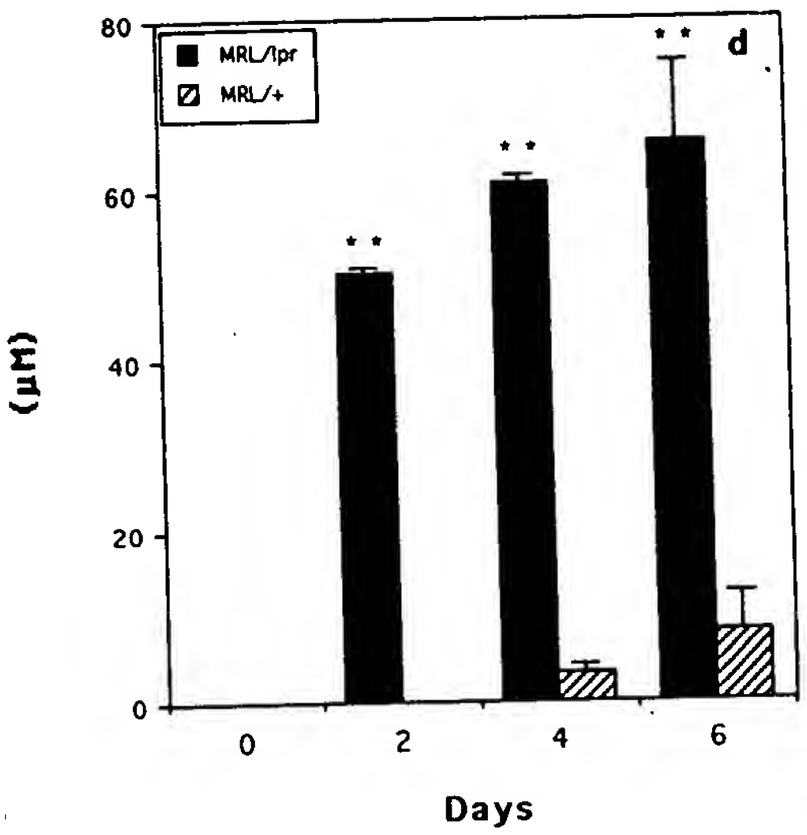
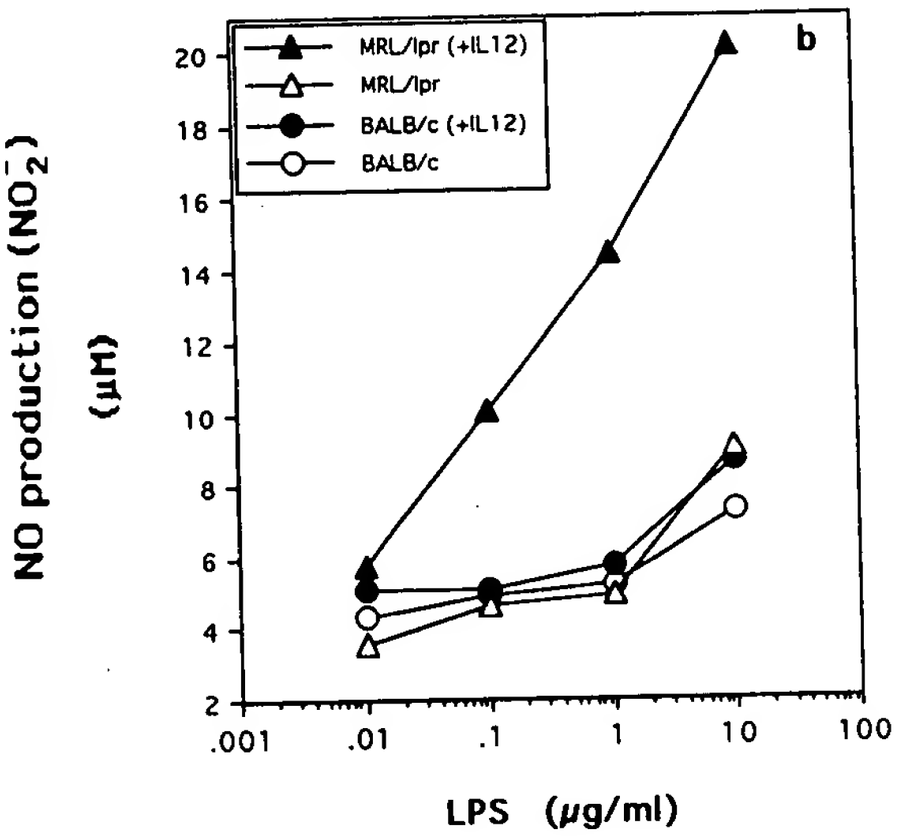
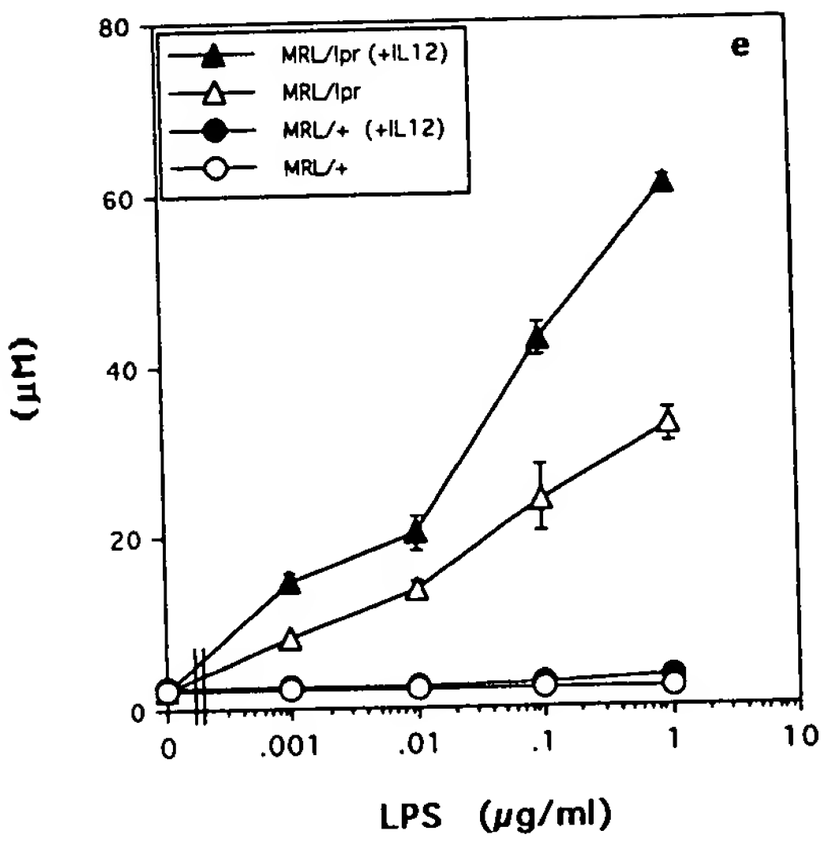


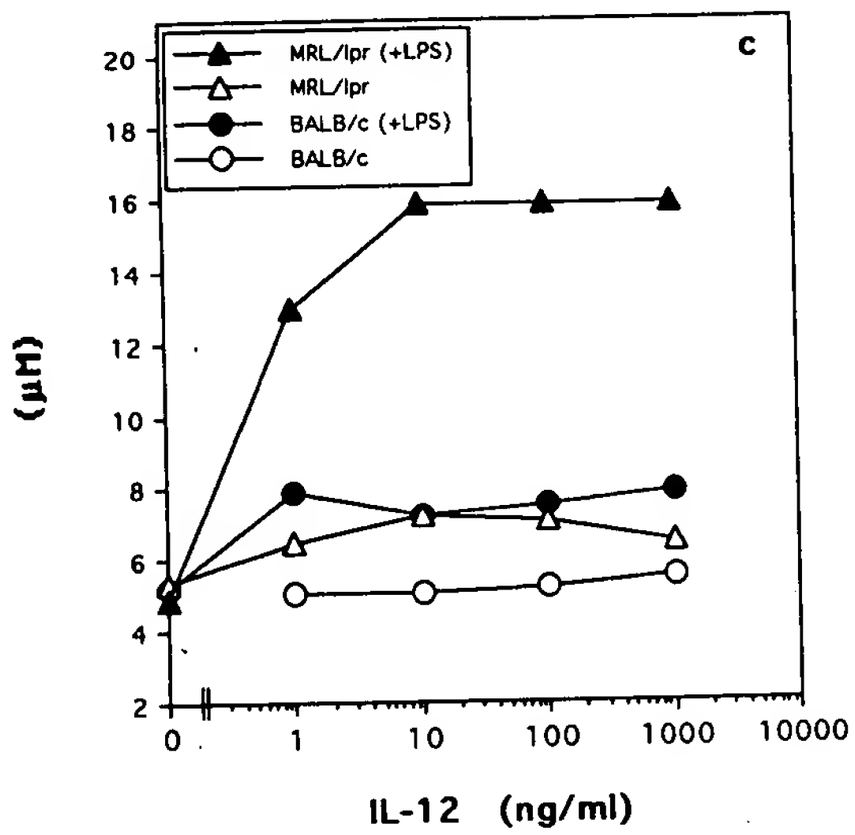
Figure 2. Enhanced IL-12 production by peritoneal cells from MRL/*lpr* mice. Pooled peritoneal cells from three mice per group of MRL/*lpr* or MRL/+ strains (2–3-mo-old). were cultured in the presence or absence of IFN-γ (50 U/ml) and LPS. IL-12 production in the cell cultures (triplicates) was determined by ELISA: (*a*) LPS dose–responses at day 4; (*b*) time course with 1 μg/ml LPS. The data shown were representative of three repeated experiments. (*c*) Western blot analysis of IL-12 expression showed inducible IL-12 p40 chain (time course) identical to that of the rIL-12 control. The two distinct p40 bands may be due to different degrees of glycosylation (24).











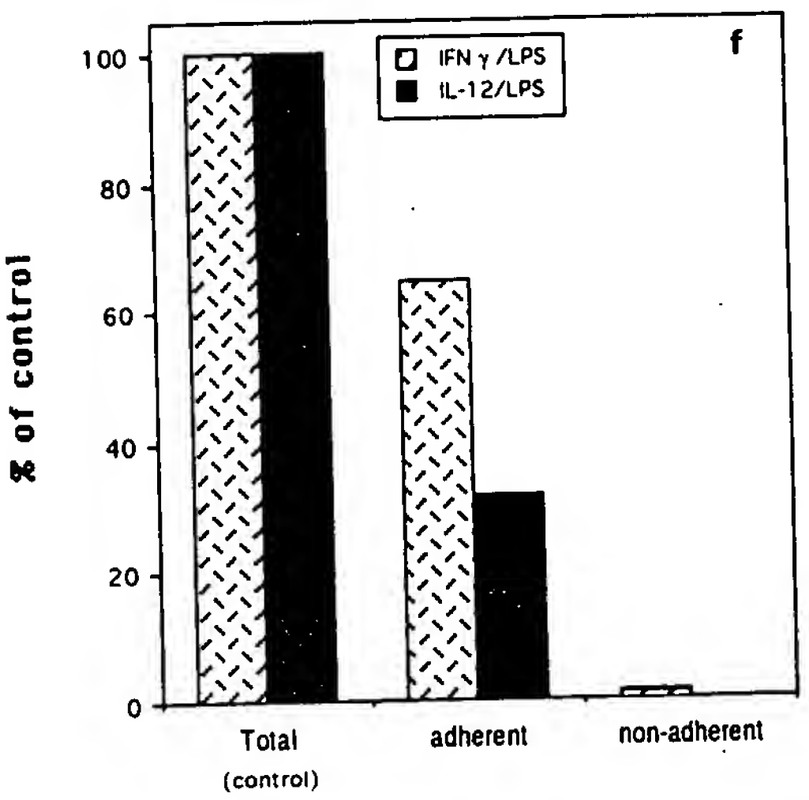


Figure 3. Spleen and peritoneal cells from MRL/*lpr* mice produced high concentrations of NO in response to rIL-12 and LPS. Pooled splenic (*a–c*) or peritoneal (*d–f*) cells from MRL/*lpr*, MRL/+, and BALB/c mice (3-mo-old, three mice per group) were stimulated in 96-well culture plates with or without fixed or different doses of rIL-12 and LPS. Culture supernatants were collected at daily intervals and nitrite levels measured by the Greiss

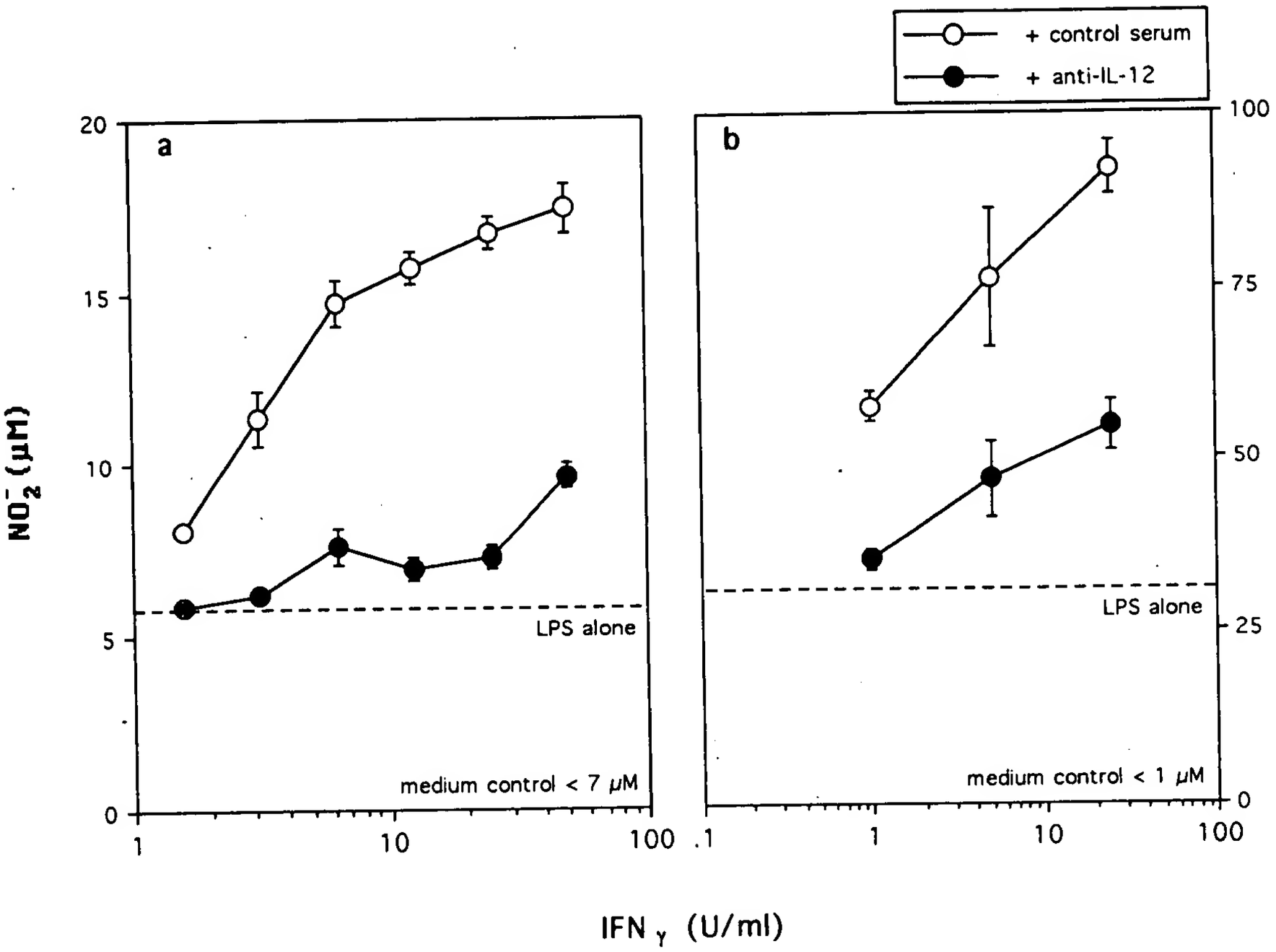


Figure 4. Effects of anti-IL-12 antibodies on IFN-γ/LPS–induced NO production by splenic and peritoneal cells from MRL/*lpr* mice. Pooled spleen (*a*) or peritoneal (*b*) cells from three MRL/*lpr* mice (3-mo-old) were stimulated for 6 or 4 d, respectively, with graded doses of IFN-γ and 1 μg/ml LPS in the presence of a rabbit anti-IL-12 antiserum (Rab.74.6) or preimmune control serum (1:100). Nitrite concentrations were expressed as mean and SD of triplicate cultures. (*Dotted lines*) Nitrite levels in cultures with LPS alone. Results from two repeated experiments were similar. (Note different scale for *a* and *b*).

tion of IL-12, a powerful immune stimulatory cytokine, by the MRL/*lpr* mice. Peritoneal cells from normal BALB/c or MRL/+ mice produced only a low level of IL-12 when cultured with IFN-γ and LPS in vitro. By contrast, peritoneal cells from age-matched *MRL/lpr* mice produced up to 10-fold more IL-12 when cultured under identical conditions (Fig. 2). IL-12 production by the cells of *MRL/lpr* mice was LPS dose- and time-dependent, reaching a plateau level after 12 h (Fig. 2, *b* and *c*). LPS or IFN-γ alone induced minimum amounts of IL-12 synthesis. Similar results were obtained with spleen cells, except that the levels of IL-12 produced were lower (data not shown).

Spleen and Peritoneal Cells from MRL/lpr Mice Produce High Levels of NO When Stimulated with IL-12 and LPS. Subsequent experiments were therefore carried out to investigate the possible link between IL-12 and NO synthesis by MRL/*lpr* mice. Spleen cells from MRL/*lpr* mice produced markedly higher concentrations of NO than those from age-matched BALB/c or MRL/+ mice when cultured with IL-12 and LPS (Fig. 3, *a–c*). Nitrite was detectable in the culture supernatant after 2 d and continued to increase up to day 6 (Fig. 3 *a*). NO production was both IL-12 and LPS dependent (Fig. 3, *b* and *c*). High concentrations of nitrite were also detected in cultures of peritoneal cells from *MRL/lpr* mice (Fig. 3, *d* and *e*). NO production in the present system is dependent on the adherent cell population (>90% macrophages) and barely detectable in the nonadherent cell population. However, removal of nonadherent cells significantly reduced (by 70–82%) the IL-12–induced NO production (Fig. 3 *f*). This suggests that the IL-12–driven NO synthesis was via its effect on nonadherent cells.

Since IFN-γ and IL-12 are known to induce each other's synthesis, we then determined whether the enhanced production of NO by spleen and peritoneal cells from *MRL/lpr* mice activated by IFN-γ and LPS was IL-12

method. (*a* and *d*) Kinetics of NO production with 1 μg/ml LPS plus 10 ng/ml rIL-12. (*b* and *e*) show LPS dose–responses with 10 ng/ml IL-12 at days 6 and 4, respectively. (*c*) IL-12 dose–response with 1 μg/ml LPS at day 6. (*f*) Induction of NO production by IL-12/LPS or IFN-γ/LPS by adherent and nonadherent peritoneal cells from MRL/*lpr* mice. Nonadherent cells were separated from adherent cells by plastic adhesion and cultured in separate wells with fixed doses of LPS (1 μg/ml) and IL-12 (10 ng/ml) or IFN-γ (50 U/ml). Data shown are nitrite concentrations as percentage of the total unseparated cells in the control cultures (mean of triplicates). ($^*P < 0.05$, $^{**}P < 0.01$).

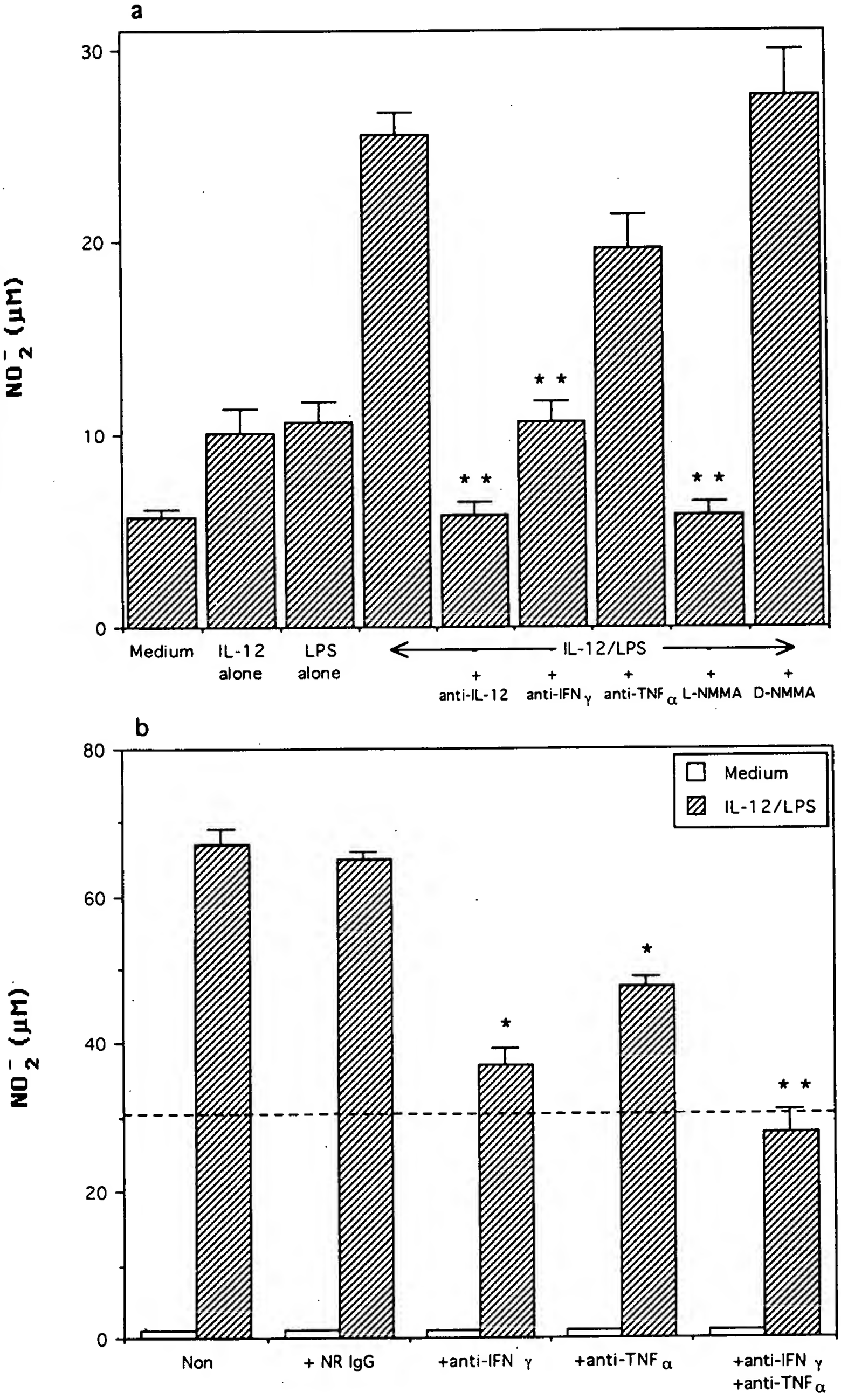


Figure 5. Inhibition of IL-12/LPS–induced NO production by antibodies to IL-12, IFN-γ, and TNF-α, and by L-NMMA. Pooled spleen (from two mice, *a*) or peritoneal (from five mice, *b*) cells from 2–3-mo-old MRL/*lpr* mice were stimulated for 6 or 4 d, respectively, with rIL-12 (10 ng/ml) and LPS (1 μg/ml) with or without addition of polyclonal antibodies (50 μg/ml) to mouse IL-12, IFN-γ, TNF-α, IFN-γ and TNF-α, or control normal rabbit IgG (*NR IgG*). In some spleen cell cultures, a NOS inhibitor, L-NMMA or its inert enantiomer control D-NMMA (500 μM) were added. Data shown are mean and SD of triplicate cultures ($^*P < 0.05$, $^{**}P < 0.01$). Similar results were obtained from four experiments. (*Dotted line*, *b*) Nitrite level in the cultures of peritoneal cells with LPS alone.

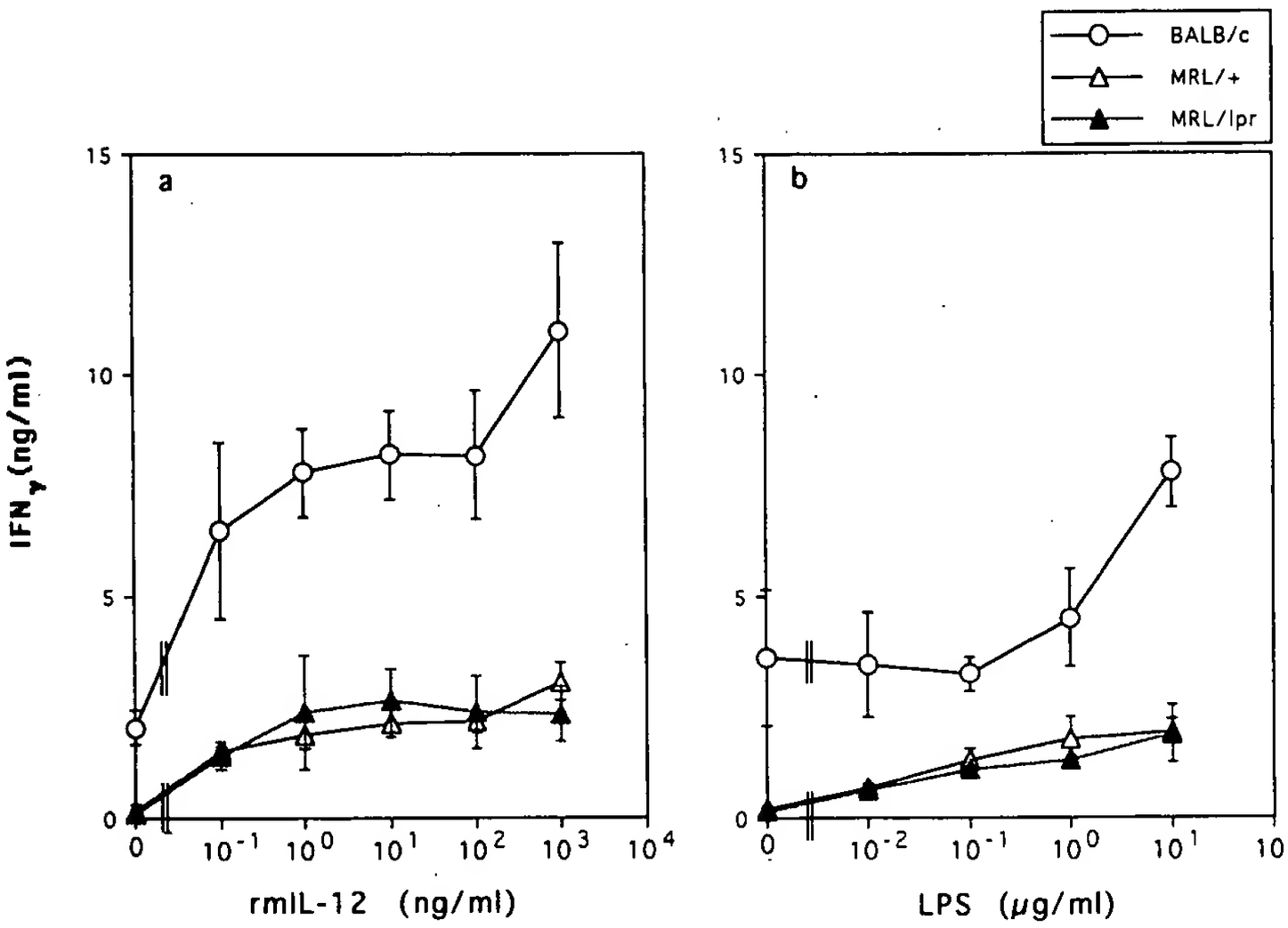


Figure 6. IL-12/LPS–induced IFN-γ production in normal and lupus mice. Pooled spleen cells from age- and sex-matched MRL/*lpr*, MRL/+, or BALB/c mice (n = 3) were stimulated with IL-12 and LPS for 3 d and IFN-γ production was determined by ELISA. (*a*) Dose–response studies of IL-12 with 1 μg/ml LPS; (*b*) dose–response studies of LPS with 10 ng/ml IL-12. IFN-γ levels in the unstimulated cultures were <35 pg/ml. Data shown are mean and SD of triplicates cultures.

dependent. Spleen and peritoneal cells were stimulated with IFN-γ and LPS as above in the presence of a rabbit anti-IL-12 antiserum. The production of NO by these cells was markedly inhibited by the antiserum but not by the control preimmune serum (Fig. 4). The inhibition was incomplete. This was because IFN-γ and LPS can be expected to directly activate macrophages to produce NO. The ability of an anti-IL-12 antibody to inhibit NO synthesis also indicated that the IL-12 detected by ELISA in the culture supernatants of cells activated with IFN-γ/LPS (e.g., Fig. 2) was not due to the IL-12 p40 homodimer.

IL-12/LPS–induced NO Production Involves IFN-γ and TNF-α. Production of NO by spleen and peritoneal cells from MRL/*lpr* mice activated with IL-12 and LPS can be completely abrogated by anti-IL-12 antibody, markedly inhibited by anti-IFN-γ antibody, and was marginally affected by anti-TNF-α antibody, but was further inhibited by the combination of anti-IFN-γ and anti-TNF-α antibodies (Fig. 5), suggesting that IL-12/LPS–induced NO synthesis may be via IFN-γ and TNF-α. However, there was no direct correlation between NO synthesis and the level of IFN-γ produced in cultures of spleen cells from MRL/*lpr*, MRL/+, or BALB/c mice when stimulated with IL-12 and LPS under identical conditions (Fig. 6, as compared to Fig. 3, *b* and *c*). Thus, whereas cells from MRL/*lpr* mice produced a high concentration of NO and those from the

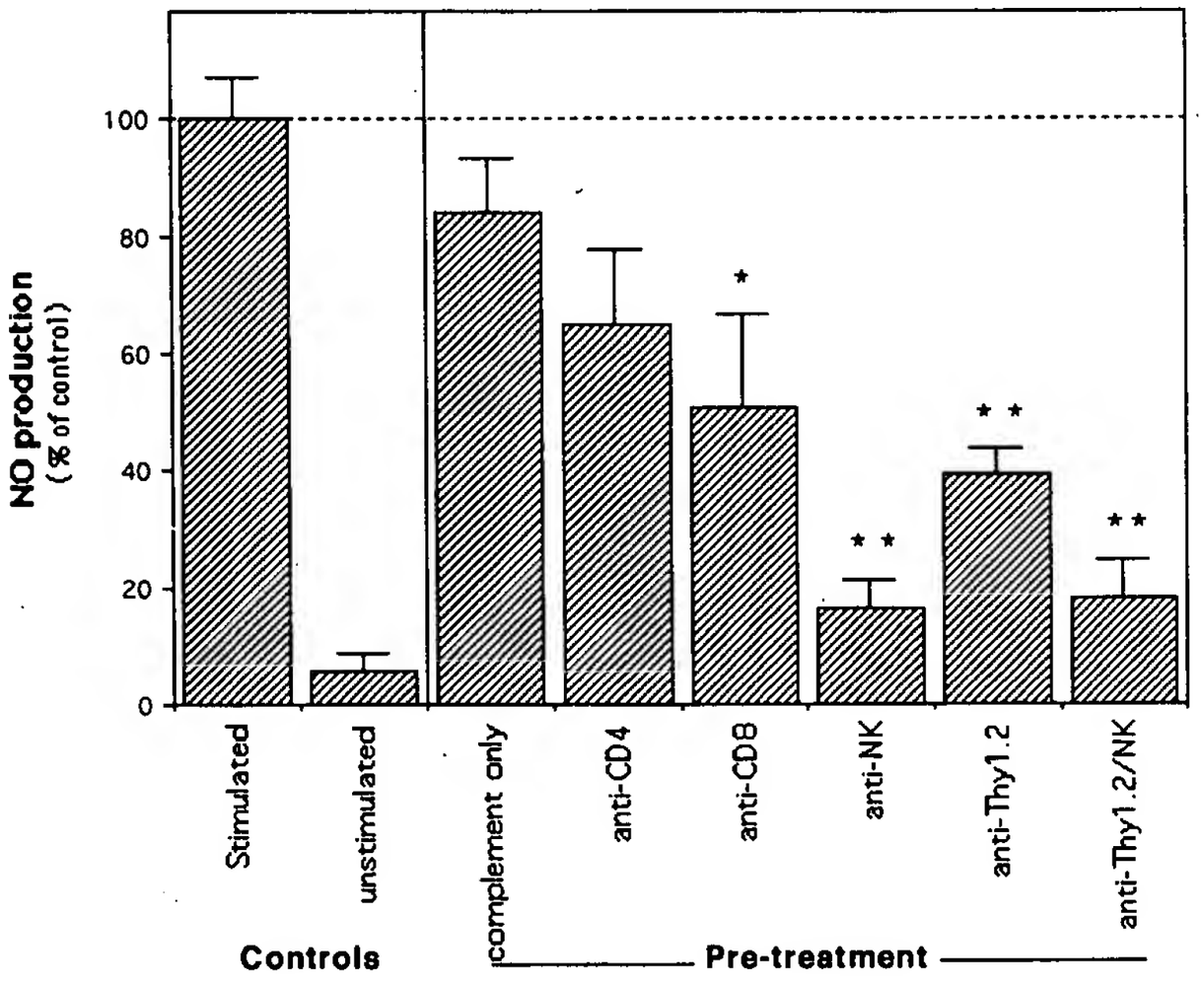


Figure 7. IL-12/LPS–induced NO synthesis involves NK and T cells. Pooled peritoneal cells from two MRL/*lpr* mice (5-mo-old) were pretreated with antibodies against CD4, CD8, NK cell marker 5E6, Thy1.2, or NK 5E6 plus Thy1.2 and then followed by incubation with rabbit serum as the source of complement. The cells were then stimulated for 4 d with rIL-12 (10 ng/ml) and LPS (1 μg/ml), or cultured in medium only as unstimulated control. Data are from one of two similar experiments and are expressed as percentage of NO concentration in the control cultures of stimulated cells without pretreatment (mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$).

MRL/+ and BALB/c mice produced only a minimum amount of NO, cells from the BALB/c mice produced markedly higher concentrations of IFN-γ than those produced by cells from MRL/*lpr* mice which was indistinguishable from those of MRL/+ mice. Spleen cells from BALB/c mice also produced markedly higher concentrations of IFN-γ and higher numbers of IFN-γ–secreting cells than those from MRL/*lpr* mice when cultured with the T cell mitogen, Con A (2.5 μg/ml), over an extended period (up to 120 h) as detected by ELISA and by ELISPOT (data not shown).

IL-12–induced NO Synthesis Involves NK and T Cells. To determine the cell types involved in the enhanced NO synthesis, cell-depletion experiments were carried out in vitro using cytolytic antibodies and complement. Depletion of $Thy1.2^+$ cells partially reduced NO production, whereas depletion of NK cells almost completely abrogated the production of NO by peritoneal cells from MRL/*lpr* mice stimulated with IL-12 and LPS (Fig. 7). Depletion of $CD4^+$ or $CD8^+$ cells alone had only a modest effect. Since some NK cells also express CD8 and Thy1.2 antigens (25), it is likely that IL-12–induced NO synthesis involves mainly NK cells in addition to the adherent population.

Evidence for the Enhanced IL-12 Synthesis in MRL/lpr Mice In Vivo. To confirm the *in vitro* observations of enhanced IL-12 activity in the lupus model, experiments were carried out to measure serum levels of IL-12, IFN-γ, and TNF-α in mice of different age and compared with those of sex- and age-matched MRL/+ and BALB/c control mice. Fig. 8 shows that serum IL-12 levels were markedly higher in the *lpr* mice, especially in the old mice with clinical disease compared with controls (12-fold in 5–8-mo-old mice). This was in parallel with the elevated levels of nitrite/nitrate in the serum (Fig. 1 *a*). Treatment of young (1–2-mo-old) mice with LPS for as little as 2 h resulted in significantly higher serum IL-12 levels in MRL/*lpr* mice compared with similarly treated control MRL/+ mice (Fig. 8 *c*). Serum IFN-γ and TNF-α were found to be low, variable, and comparable (data not shown).

rIL-12 Accelerates Autoimmune Disease in MRL/lpr Mice. To investigate directly the role of IL-12 in the induction of autoimmune disease, young (3-wk-old) MRL/*lpr* mice were given daily intraperitoneal injections of rIL-12 (300 ng/mouse/day) or a similar volume of PBS for 9 wk. Mice were then killed and the histopathology of the kidney examined. Gross morphology of the kidneys from the IL-12–treated mice had a pale waxy surface and were firmer on sectioning, whereas those from the control PBS-injected mice appeared normal. Histological examination revealed enlarged glomeruli with significant glomerular and mesangial hypercellularity in the IL-12–treated group. In particular, most of the IL-12–treated group showed severe damage to the glomeruli, with thickening of the Bowman's capsule basement membrane and tuft-to-capsule adhesions and protein casts (Fig. 9 *c*) which were largely absent or scanty in the PBS-treated group (Fig. 9 *a*). In contrast, pyelonephritis with extensive vasculitis and infiltration of mononuclear cells at the kidney medullary region (Fig. 9 *b*) was prominent in all the mice in the PBS-treated group. These changes were minimal in the IL-12–treated mice (Fig. 9 *d*).

Spleen cells from the rIL-12–treated mice showed a significantly higher percentage of $CD3^+$ (51.5 vs 44.5%, $P = 0.02$, $n = 5$), $CD8^+$ (9.2 vs 5.3%, $P = 0.02$) and double negative (22.6 vs 17.3%, $P = 0.02$) T cells than those from untreated mice. There was, however, no significant difference in the spleen weight or the percentage of $CD4^+$ T cells in the spleen cell populations between the two groups. There was also no significant difference in the anti-ss or ds DNA antibody (total antibody as well as IgM and IgG isotype) concentrations in the serum between treated and untreated mice (data not shown). However, serum IFN-γ (16.5 ± 3.8 vs 5.2 ± 1.4 pg/ml, $P = 0.0189$) and nitrite/nitrate (54.2 ± 2.6 vs 29.6 ± 3.0 μM, $P = 0.0304$) were elevated in the IL-12–treated mice compared with those of PBS-treated mice.

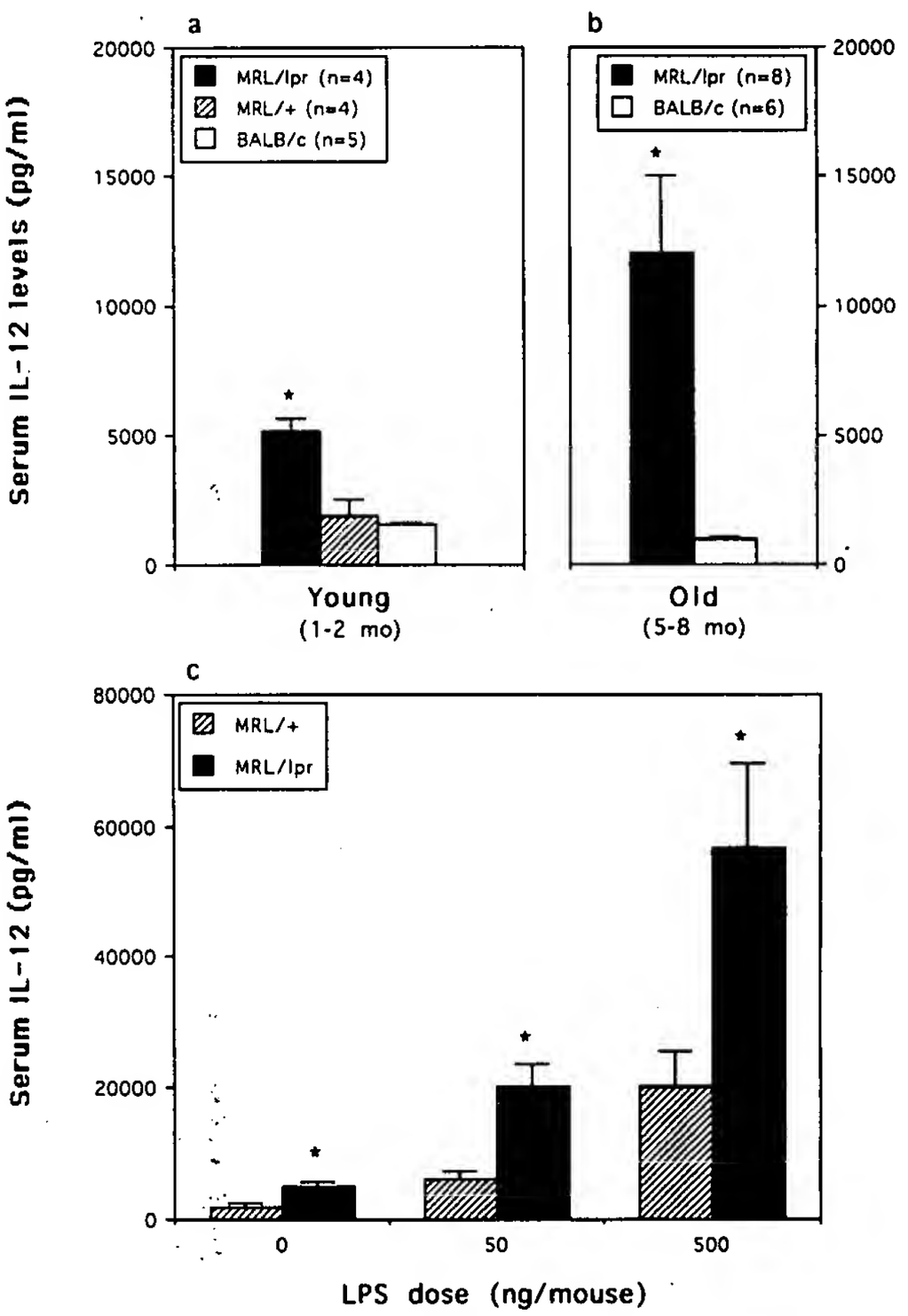
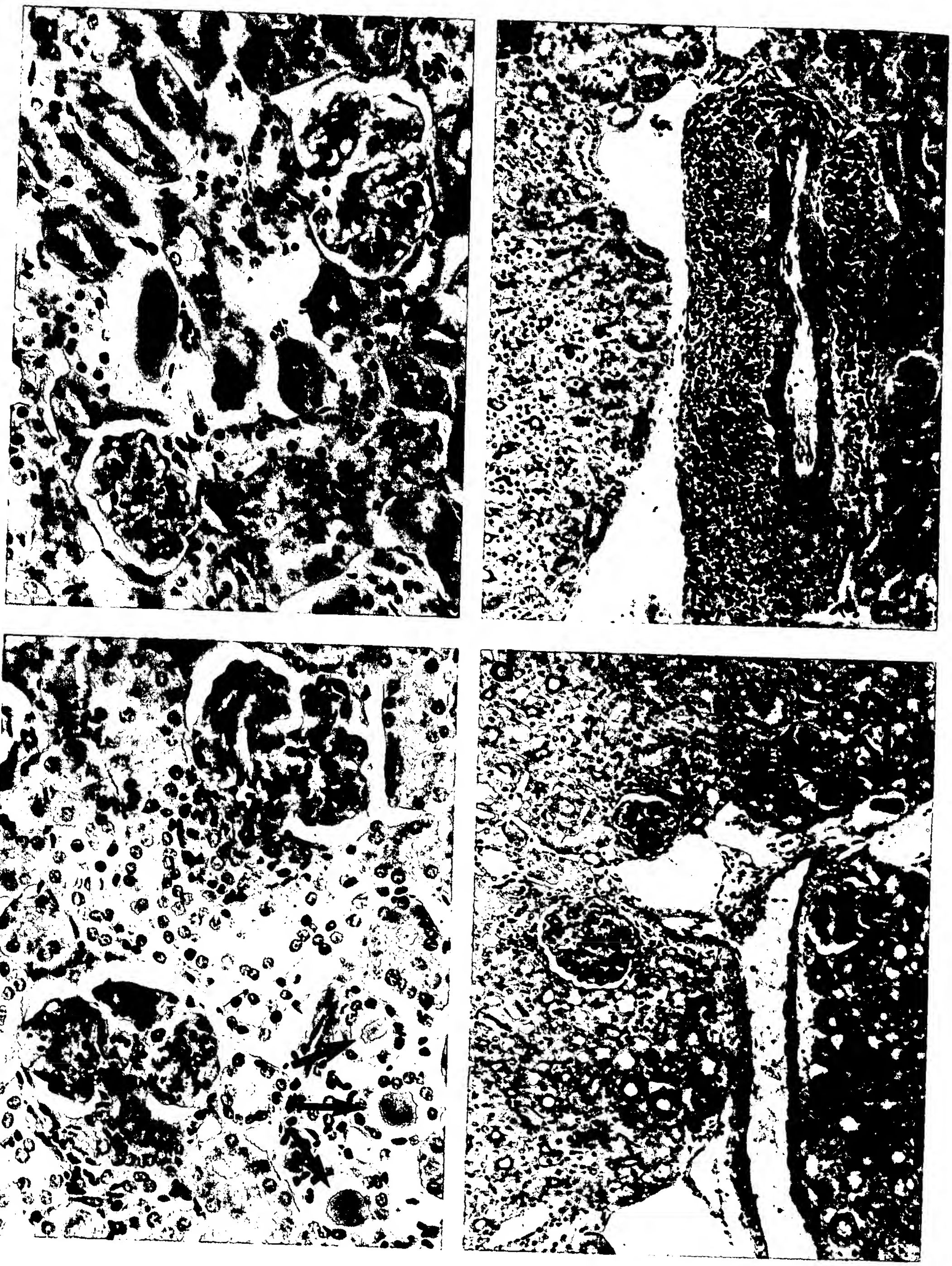


Figure 8. Elevated serum IL-12 levels in untreated and LPS-treated MRL/*lpr* mice. Serum IL-12 levels in untreated (*a*) young (1–2 mo) and (*b*) old (5–8 mo), and in (*c*) LPS-treated young (1–2 mo) MRL/*lpr* mice. Control mice were sex- and age-matched MRL/+ or BALB/c mice. For the LPS treatment, a total of six groups of mice (four per group) were injected (i.v. tail vein) with 50 or 500 ng per mouse of LPS in 0.1 ml PBS or PBS alone as indicated. Serum samples were collected 2 h after the treatment and IL-12 levels were determined. All samples were assayed individually by ELISA; the statistical significance of differences between lupus and the control strains of the same age groups is indicated ($^*P < 0.05$).



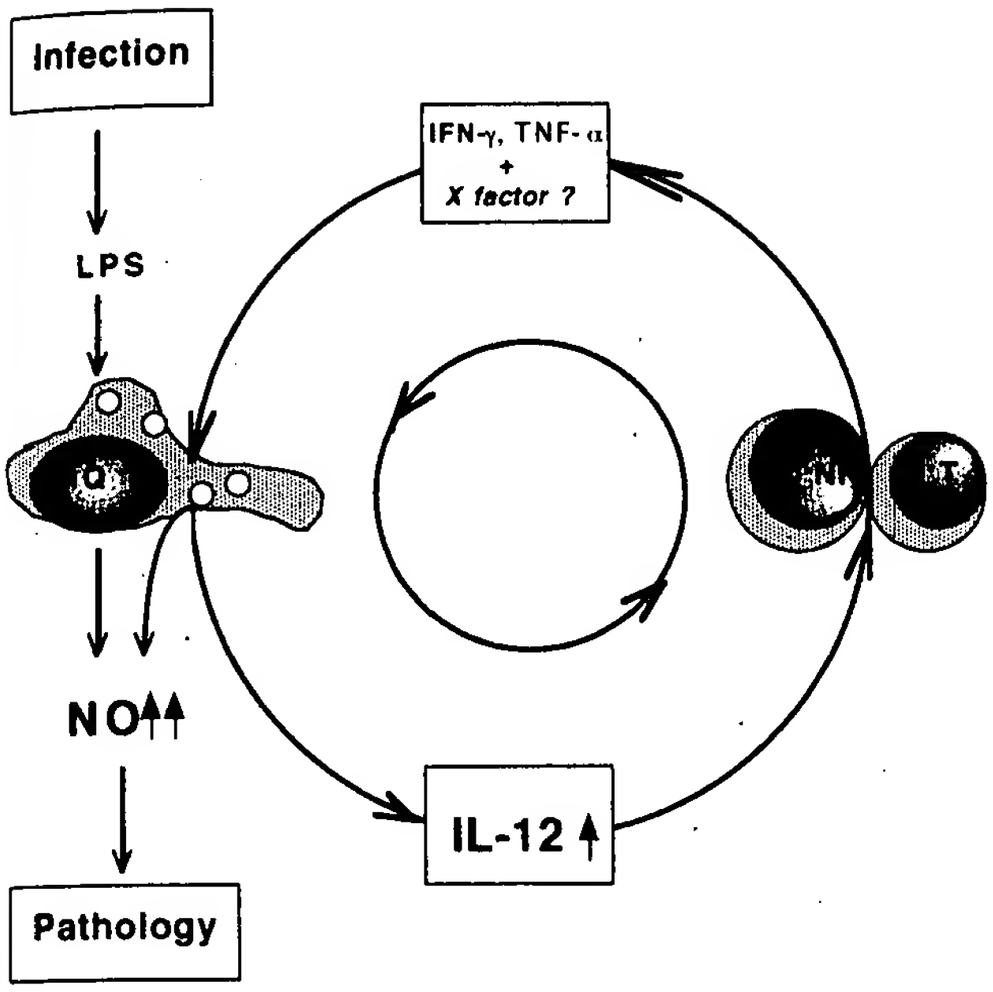


Figure 10. Schematic representation of the possible mechanism for the induction of renal pathology (glomerulonephritis) in MRL/*lpr* mice by enhanced IL-12 production and NO synthesis (for details see text).

It is interesting to note that treatment of MRL/+ mice with IL-12 did not induce any detectable renal pathology (glomerulonephritis or proteinuria, data not shown), suggesting that MRL/*lpr* mice are genetically predisposed to the effect of IL-12. However, treatment of MRL/*lpr* mice (starting age: 2 mo) with a sheep anti–mouse IL-12 antibody (sheep no. 7, 100 μg/mouse i.p., weekly for 7 wk) led to a initial reduction of proteinuria followed by a rebound (data not shown) possibly due to increased formation of immune complexes resulting from repeated injection of foreign proteins.

Discussion

Data presented here demonstrate that serum from MRL/*lpr* mice contained significantly higher concentrations of IL-12 with or without treatment with LPS in vivo compared with those of similarly treated control MRL/+ mice. In addition, spleen and peritoneal cells from MRL/*lpr* mice produced significantly higher concentrations of IL-12 than MRL/+ or BALB/c mice in response to activation by IFN-γ and LPS in vitro. Furthermore, cells from MRL/*lpr* mice were more responsive to IL-12 and LPS, producing higher concentrations of NO than those from the control MRL/+ mice. Finally, daily injection of rIL-12 led to accelerated glomerulonephritis in the MRL/*lpr* mice but not in the MRL/+ mice.

These results suggest a causal relationship between enhanced capacity to produce IL-12 and the spontaneous autoimmune disease in this model of SLE as depicted schematically in Fig. 10. An earlier report (5) demonstrated that NO is a critical mediator of the autoimmune disease in MRL/*lpr* mice. It has also been documented that many autoimmune animal models including the lupus MRL strain of mice do not develop autoimmune disease when kept in a germ-free environment (26, 27), consistent with our finding that LPS and IFN-γ are required for the activation of macrophages to produce high concentrations of NO and IL-12, which is produced by monocyte/macrophages (28). In contrast to normal MRL/+ or BALB/c mice, the production of NO in the MRL/*lpr* mice is further exaggerated by the high concentration of IL-12 produced by activated macrophages. IL-12 activates NK and T cells to produce IFN-γ and perhaps other yet unidentified factor(s) which, together with LPS, further enhance NO synthesis. This cycle of amplification produces exaggerated levels of NO leading to the pathology. This is consistent with the delayed onset of NO synthesis in the cultures activated with IL-12 (Fig. 3, requiring 4–6 d for optimal production of NO). The activation of NK and T cells by IL-12 for the production of IFN-γ has been well documented (29–31). However, in the present system, there was a lack of direct correlation between enhanced NO synthesis and IFN-γ production by MRL/*lpr* and control MRL/+ and BALB/c mice. Nevertheless, IFN-γ and TNF-α were required for IL-12–driven NO synthesis. It is therefore likely that an additional factor(s) produced by IL-12–activated NK or T cells is required to synergize with IFN-γ for the production of high concentrations of NO. NK cell activity is known to be altered in MRL/lpr mice. However, this was based on their lytic activity rather than their activity to produce IFN-γ.

IL-12 is essential for the differentiation of the Th1 subset of T cells (32–34). It is also a powerful adjuvant for the induction of protective immunity against diseases such as cutaneous leishmaniasis (35) in which Th1 cells are the main protective mechanism (for reviews see references 36, 37). Our results indicate that excessive production of IL-12 or the administration of rIL-12 can cause autoimmune disease in susceptible mice, demonstrating the negative side of the therapeutic use of IL-12. This is consistent with a number of recent reports showing that administration of IL-12 induced: (*a*) earlier onset of insulin-dependent diabetes mellitus in female NOD mice (38); (*b*) more severe and prolonged

Figure 9. Effects of IL-12 treatment of MRL/*lpr* mice on renal pathology. Photomicrographs of kidney sections from MRL/*lpr* mice (3-mo-old) showing two essentially normal glomeruli in the kidney cortex of a PBS-treated control mouse (*a*); and severe glomerulonephritis in the cortical region of a IL-12–treated mouse, including structural damage to two enlarged glomeruli with hypercellularity and adhesion (*c*), as well as protein casts in the tubules (*c* and *d*, *arrows*). In contrast, histological examination of the medullary region shows severe pyelonephritis in the PBS-treated mouse (*b*) featuring extensive mononuclear cell infiltration in the perivascular interstitium, which is, however, markedly reduced in the IL-12–treated mouse (*d*). Periodic Acid-Schiff stain, ×250 (*a* and *c*), ×100 (*b* and *d*).

disease in adoptively transferred experimental allergic encephalomyelitis (39); and (*c*) destructive collagen-induced arthritis (40). Our study here demonstrates that the pathogenic effect of IL-12 in the lupus model is likely to be due to the increased production of NO. This finding not only advances our knowledge of the pathogenesis of this lupus model (and by extension to SLE), it also suggests two potential means of therapeutic intervention of the progression of this disease: neutralization of IL-12 or inhibition of iNOS.

Glomerulonephritis is a severe complication of the renal involvement which is the major cause of pathology and death in SLE (18, 41). Although it is generally believed that the renal pathology is due to autoantibody production, immune complex deposition and complement activation, slow infections have been shown to play an important role in triggering these autoimmune responses in many models of autoimmune disorders (26). We observed here that IL-12–treated mice had clearly reduced pyelonephritis which is known to be commonly induced by infections (42). Our results suggest that IL-12 might have strengthened the host's defense against infection in these mice which are otherwise immunodeficient (43–45). Thus, treatment of the autoimmune disease aiming at neutralization of IL-12 may weaken the host immune response, leading to uncontrolled infection. These results therefore demonstrated that IL-12 is beneficial in controlling infections. However, excessive production of IL-12, as in the lupus mice, will lead to severe immunopathology.

MRL/*lpr* mice differ from the MRL/+ mice in the impairment of transcription of the gene encoding Fas antigen by insertion of a transposable element into the second intron of the gene (46). However, *lpr* is not a null mutation and the inhibition of Fas expression is incomplete (47). The relationship between the impaired *fas* gene expression and enhanced IL-12 and NO production by the MRL/*lpr* mice is at present unclear, but amenable to experimental investigation.

We thank Dr. X.-q. Wei for helpful discussion, Mr. N. Whyte for technical assistance on histopathology, and the Genetic Institute for the generous gift of rIL-12 and anti-IL-12 antibodies.

This study was supported by the Wellcome Trust and the Robertson Trust.

Address correspondence to Dr. Foo Y. Liew, Department of Immunology, University of Glasgow, Western Infirmery, Glasgow G11 6NT, United Kingdom.

Received for publication 15 September 1995 and in revised form 27 December 1995.

References

1. Cohen, P.L., and R.A. Eisenberg. 1991. *Lpr and gld*: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol.* 9:243–269.
2. Andrew, B.S., R.A. Eisenberg, A.N. Theofilopoulos, S. Izui, C.B. Wilson, P.J. McConahey, E.D. Murphy, J.B. Roths, and F.J. Dixon. 1978. Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. *J. Exp. Med.* 148:1198–1215.
3. Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature (Lond.).* 356:314–317.
4. Watson, M.L., J.K. Rao, G.K. Gilkeson, P. Ruiz, E.M. Eicher, D.M. Pisetsky, A. Matsuzawa, J.M. Rochelle, and M.F. Seldin. 1992. Genetic analysis of MRL-*lpr* mice: relationship of the Fas apoptosis gene to disease manifestations and renal disease modifying loci. *J. Exp. Med.* 176:1645–1656.
5. Weinberg, J.B., D.L. Granger, D.S. Pisetsky, M.F. Seldin, M.A. Misukonis, S.N. Mason, A.M. Pippen, P. Ruiz, E.R. Wood, and G.S. Gilkeson. 1994. The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease expression in MRL-*lpr/lpr* mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered N^G-monomethyl-L-arginine. *J. Exp. Med.* 179:651–660.
6. Moncada, S., R.M.J. Palmer, and E.A. Higgs. 1991. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* 43:109–142.
7. Nathan, C.F., and J.B. Hibbs, Jr. 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.* 3:65–70.
8. Liew, F.Y., and F.E.G. Cox. 1991. Non-specific defence mechanism: the role of nitric oxide. *Immunol. Today.* A17–21.
9. Marletta, M.A. 1993. Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* 268:12231–12234.
10. Bredt, D.S., and S.H. Snyder. 1994. Nitric oxide: a physiologic messenger molecule. *Annu. Rev. Biochem.* 63:175–195.
11. Nathan, C., and Q.-w. Xie. 1994. Nitric oxide synthase: roles, tolls, and control. *Cell.* 78:915–918.
12. Vladutiu, A.O. 1995. Role of nitric oxide in autoimmunity. *Clin. Immunol. Immunopathol.* 76:1–11.
13. Liew, F.Y. 1995. Regulation of lymphocyte functions by nitric oxide. *Curr. Opin. Immunol.* 7:396–399.
14. Palmer, R.M.J., D.S. Ashton, and S. Moncada. 1988. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature (Lond.).* 333:664–666.
15. Kwon, N.S., C.F. Nathan, C. Gilker, O. Griffith, D. Mathews, and D.J. Stuehr. 1990. L-citrulline production from L-arginine by macrophage nitric oxide synthase; the ureido oxygen derives from dioxygen. *J. Biol. Chem.* 265:13442–13445.
16. Leone, A.M., R.M.J. Palmer, R.G. Knowles, P.L. Francis,

D.S. Ashton, and S. Moncada. 1991. Constitutive and inducible nitric oxide synthases incorporate molecular oxygen into both nitric oxide and citrulline. *J. Biol. Chem.* 266:23790–23795.

17. Nathan, C., and Q.-W. Xie 1994. Regulation of biosynthesis of nitric oxide. *J. Biol. Chem.* 269:13725–13728.
18. Theofilopoulos, A.N. 1992. Murine models of lupus. *In* Systemic Lupus Erythematosus. 2nd ed., Section II. R.G. Lahita, editor. Churchhill Livingstone, Inc., New York. 121–194.
19. Umland, S., R. Lee, M. Howard, and C. Martens. 1989. Expression of lymphokine genes in splenic lymphocytes of autoimmune mice. *Mol. Immunol.* 26:649–656.
20. Hutchings, P.R., G. Cambridge, J.P. Tite, A. Meager, and A. Cooke. 1989. The detection and enumeration of cytokine-secreting cells in mice and man and the clinical application of these assays. *J. Immunol. Methods.* 120:1–8.
21. Palmer, R.M.J., A.G. Ferrige, and S. Moncada. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature (Lond.).* 327:524–526.
22. Green, L.C., D. Wagner, J. Glogowski, P.L. Skipper, J.J. Wishnok, and S.R. Tannenbaum. 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal. Biochem.* 126:131–138.
23. Luzuy, S., J. Merino, H. Engers, S. Izui, and P.-H. Lumbert. 1986. Autoimmunity after induction of neonatal tolerence to alloantigens: role of B cell chimaerism and F1 donor B cell activation. *J. Immunol.* 136:4420–4426.
24. Podlaski, F.J., V.B. Nanduri, J.D. Hulmes, Y.E. Pan, W. Levin, W. Danho, R. Chizzonite, M.K. Gately, and A.S. Stern. 1992. Molecular characterization of interleukin 12. *Arch. Biochem. Biophys.* 294:230–237.
25. Trinchieri, G. Biology of natural killer cells. 1989. *Adv. Immunol.* 47:187–376.
26. Rook, G.A.W., P.M. Lydyard, and J.L. Stanford. 1993. A reappraisal of the evidence that rheumatoid arthritis and several other idiopathic diseases are slow bacterial infections. *Ann. Rheum. Dis.* 52:S30–S38.
27. Taurog, J.D., J.A. Richardson, J.T. Croft, W.A. Simmons, M. Zhou, J.L. Fernandezsueiro, E. Balish, and R.E. Hammer. 1994. The germ-free state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *J. Exp. Med.* 180:2359–2364.
28. D'Andrea, A., M. Rengaraju, N.M. Valiante, J. Chehimi, M. Kubin, M. Aste, S.H. Chan, M. Kobayashi, D. Young, E. Nickbarg et al. 1992. Production of natural killer cells stimulatory factor (IL-12) by peripheral blood mononuclear cells. *J. Exp. Med.* 176:1387–1398.
29. Chan, S.H., B. Perussia, J.W. Gupta, M. Kobayashi, M. Pospisil, H.A. Young, S.F. Wolf, D. Young, S.C. Clark, and G. Trinchieri. 1991. Induction of interferon γ production by natural killer cell stimulatory factor: characterisation of the responder cells and synergy with other inducers. *J. Exp. Med.* 173:869–879.
30. Tripp, C.S., S.E. Wolf, and E.R. Unanue. 1993. Interleukin-12 and tumour necrosis factor α are costimulators of interferon-γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis and interleukin-10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. USA.* 90:3725–3729.
31. Gazzinelli, R.T., S. Hieny, T.A. Wynn, S. Wolf, and A. Sher. 1993. Interleukin-12 is required for the T-lymphocyte–independent induction of interferon-γ by an intracellular parasite and induces resistance in T-cell deficient hosts. *Proc. Natl. Acad. Sci. USA.* 90:6115–6119.
32. Hsieh, C.S., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Development of Th1 $CD4^+$ T cells through IL-12 produced by Listeria-induced macrophages. *Science (Wash. DC).* 260:547–549.
33. Seder, R.A., R. Gazzinelli, A. Sher, and W.E. Paul. 1993. Interleukin-12 acts directly on $CD4^+$ T cells to enhance priming for interferon-γ production and diminishes interleukin-4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA.* 90:10188–10192.
34. Manetti, R., P. Parronchi, M.G. Guidizi, M.P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin-12) induces T-helper type 1 (Th1)–specific immune response and inhibits the development of IL-4–producing cells. *J. Exp. Med.* 177:1199–1204.
35. Afonso, L.C.C., T.M. Scharton, L.Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott. 1994. The adjuvant effect of interleukin-12 in a vaccine against Leishmania major. *Science (Wash. DC).* 263:235–237.
36. Liew, F.Y., and C.A. O'Donnell. 1993. Immunology of Leishmaniasis. *Adv. Parasitol.* 32:161–259.
37. Reiner, S.L., and R.M. Locksley. 1995. The regulation of immunity to Leishmania major. *Annu. Rev. Immunol.* 13: 151–177.
38. Trembleau, S., G. Penna, E. Bosi, A. Mortara, M.K. Gately, and L. Adorini. 1995. Interleukin-12 administration induces T-helper type-1 cells and accelerates autoimmune diabetes in NOD mice. *J. Exp. Med.* 181:817–821.
39. Leonard, J.P., K.E. Waldburger, and S.J. Goldman. 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J. Exp. Med.* 181:381–386.
40. Germann, T., J. Szeliga, H. Hess, S. Storkel, F.J. Podlaski, M.K. Gately, E. Schmitt, and E. Rude. 1995. Administration of interleukin-12 in combination with type-II collagen induces severe arthritis in DBA/1 mice. *Proc. Natl. Acad. Sci. USA.* 92:4823–4827.
41. Corriea, P., J.S. Cameron, J.D. Lian, J. Hicks, C.S. Ogg, D.G. Williams, C. Chantler, and D.G. Haycock. 1985. Why do patients with lupus nephritis die? *Br. Med. J.* 290:126–131.
42. Brumfit, W., and A. Percival. 1964. Pathogenesis and laboratory diagnosis of non-tuberculous urinary tract infection. *J. Clin. Pathol.* 17:482–491.
43. Gutierrez-Ramos, J.C., J.L. Andreu, Y. Revilla, E. Vinuela, and C. Martinez-A. 1990. Recovery from autoimmunity of MRL/*lpr* mice after infection with an interleukin-2/vaccinia recombinant virus. *Nature (Lond.).* 346:271–274.
44. Huang, F.-P., and D.I. Stott. 1993. Restoration of an early, progressive defect in responsiveness to T-cell activation in lupus mice by exogenous IL-2. *Autoimmunity.* 15:19–29.
45. Pelton, B.K., and A.M. Denman. 1982. Defective B-cell function in systemic lupus erythematosus. *Clin. Exp. Immunol.* 48:513–518.
46. Adachi, M., R. Watanabe-Fukunaga, and S. Nagata. 1993. Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice. *Proc. Natl. Acad. Sci. USA.* 90:1756–1760.
47. Kobayashi, S., T. Hirano, M. Kakinuma, and T. Uede. 1993. Transcriptional repression and differential splicing of Fas messenger-RNA by early transposon (ETN) insertion in autoimmune lpr mice. *Biochem. Biophy. Res. Commun.* 191:617–624.

STIC-ILL

From: Prasad, Sarada
Sent: Tuesday, March 06, 2001 5:33 PM
To: STIC-ILL

Please fetch me the following full text citations for the case # 09,395,038

1. Philosophical transactions of the roayal Society of London B Biological Sciences (Sept. 29, 1997) vol. 352, No. 1359, pages 1311-1315.(cytokines and nitric oxide as effector molecules against parasitic infections.
2. J Immunol. 1994, vol.153, No.11, pages 5200-5209. (Elevated expression of Th1 cytokines and nitric oxide synthase in the lungs of vaccinated mice after challenge infection with schistosoma mansoni.)
3. Cancer immunol, Immunotherpy, 1996 jan, vol.42, No.1, pages 38-46. (Effects of N-methyl arg, an inhibitor of nitric oxide synthesis on IL-2 induced capillary leakage and anti-tumor responses innhealthy and tumor bearing mice.
4. Infection and immunity 1997, vol. 65, No. 4 1307-1312. (Expression of cytokines and inducible nitric oxuide synthase mRNA in the lungs of mice infected with cryptococcus neoformans: effects of interleukin 12).
5. Journal of general virology 1998, vol.79, No. 4, pages 825-830 (Mice lacking inducible niric oxide synthase are more susceptible to herpers simplex virus infection despite enhanced Th1 cells responses)
6. J Neuroimmunology 1996, vol.68, No. 1-2, pages 101-108. (Interferon gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons.)
7. J Experimental medicine 1996, vol.183, No. 4, pages 1447-1459. (The role of interleukin 12 and nitric oxide in the development of spontaneous auroimmune disese in mrl...mice).

thank you,

Sarada Prasad, Ph.D.
Patent Examiner
Art Unit 1646
Room CM1-8B17
Tel: 703-305-1009

Journal of General Virology (1998), **79**, 825–830. Printed in Great Britain

Mice lacking inducible nitric-oxide synthase are more susceptible to herpes simplex virus infection despite enhanced Th1 cell responses

Alasdair MacLean,[1] Xiao-Qing Wei,[2] Fang-Ping Huang,[2] Umaima A. H. Al-Alem,[3] Woon Ling Chan[3] and Foo Y. Liew[2]

[1,2] Division of Virology[1] and Department of Immunology[2], University of Glasgow, Glasgow G11 6NT, UK

[3] Department of Virology, St Bartholomew's Hospital and Royal London Hospital School of Medicine, University of London, London EC1A 7BE, UK

Mice deficient in the inducible nitric-oxide synthase (iNOS), constructed by gene-targeting, were significantly more susceptible to herpes simplex virus (HSV)-1 infection, displayed a delayed clearance of virus from the dorsal root ganglia (DRG) and exhibited an increase in the frequency of virus reactivation in DRG compared with similarly infected heterozygous mice. The infected iNOS-deficient mice developed enhanced Th1-type immune responses and their spleen cells produced higher concentrations of IL-12 than similarly infected heterozygous mice. This finding suggests that iNOS plays an important role in resistance against HSV-1 infection. Furthermore, nitric oxide (NO) may block the development of Th1 cells via inhibition of IL-12 synthesis and thereby play a role in immune regulation.

Introduction

Inducible nitric-oxide synthase (iNOS) catalyses the synthesis of high concentrations of nitric oxide (NO) from L-arginine and plays a role in microbicidal and tumoricidal activities, and in immunopathology (Moncada & Higgs, 1993; Nathan & Xie, 1994). It may also be important in immune regulation (Albina *et al.*, 1991; Liew, 1995). The role of NO in virus infection is, however, controversial (Croen, 1993; Karupiah *et al.*, 1993; Burkrinsky *et al.*, 1995; Mannick *et al.*, 1994; Rolph *et al.*, 1996; Adler *et al.*, 1997). Most of these studies used L-arginine analogues which are competitive inhibitors of NO synthases. The discrepancy between the results may be attributable to the non-isoform-selective nature and the variation in the bioavailability of the inhibitors. By gene targeting, we have constructed a mouse strain lacking iNOS (Wei *et al.*, 1995). These mice are highly susceptible to intracellular parasitic infection. We have now tested the ability of these mice to resist herpes simplex virus (HSV)-1 infection.

Methods

■ **Mice.** iNOS-deficient mice were generated as described previously (Wei *et al.*, 1995). Disruption of the murine iNOS gene was achieved by homologous recombination in 129sv embryonic stem cells. The recombinant allele was passed through the germ line following mating of embryonic stem cell chimaeras with MF1 (Harlan Olac). The homozygous, heterozygous and wild-type mice thus generated were backcrossed to MF1 for three generations. All the mice used were from matings of littermates and should therefore have had a similar MF1 gene background. Peritoneal cells from mutant mice did not produce iNOS protein following activation by IFN-γ and LPS *in vitro* as judged by Western blot. They also did not produce detectable amounts of NO following up to 48 h culture with IFN-γ plus LPS. By 72 h, however, a low level of nitrite was detectable in the culture supernatant of cells from mutant mice. This may reflect the accumulation of nitrite produced by constitutive NOS, or the induction of constitutive NOS (Wei *et al.*, 1995). Female mutant mice and their heterozygous littermates were used at 3–4 weeks old. Extensive previous experiments showed no significant phenotypic difference between the heterozygous and wild-type mice (Wei *et al.*, 1995); hence in the present study only heterozygous mice were used as controls.

■ **Virus and infection.** HSV-1 (Glasgow strain 17^+) was grown and titrated in baby hamster kidney cells (BHK21/C13) (MacPherson & Stoker, 1962) propagated in Eagle's medium as described previously (MacLean *et al.*, 1991). Mice were inoculated in the right hind footpad with the appropriate doses of virus (Robertson *et al.*, 1992), which were titrated prior to inoculation.

■ **Peripheral virulence.** Groups of five mice were inoculated with a series of 10-fold dilutions of virus. Mice were examined daily and the LD_{50} calculated according to the formula of Reed & Muench (1938) on the basis of the number of deaths up to 42 days post-infection.

■ **Virus replication during acute infection.** Mice were inoculated with 10^5 p.f.u. of virus per mouse. Virus replicates in the footpad, enters

Author for correspondence: Foo Y. Liew.
Fax +44 141 337 3217. e-mail f.y.liew@clinmed.gla.ac.uk

0001-5188

the sciatic nerve and travels by retrograde transport to the dorsal root ganglion (DRG) where replication occurs prior to the establishment of a latent infection. Groups of mice were sacrificed at regular intervals and the right rear footpad together with the two lowest thoracic (T11, T12), five lumbar (L1–5) and three sacral (S1–3) DRG were removed from the inoculated side and stored at −70 °C. All ten ganglia from each mouse were pooled, but the footpad and DRG from each mouse were processed separately. Following homogenization and sonication, the virus yield was determined by titration in BHK cells (Robertson *et al.*, 1992).

■ **Reactivation of latent virus from DRG.** Mice were inoculated with 10^4 or 10^5 p.f.u. of virus per mouse and examined daily for clinical symptoms. Mice surviving 6 weeks were assayed for the presence of latent virus. The mice were sacrificed and DRG removed from the inoculated side, placed individually in culture medium in microtitre dishes and screened for the release of infectious virus every second day by transferring the culture medium to control BHK cells which were then incubated for 2 days at 37 °C before examining for the presence of virus cytopathic effect.

■ **Antibody titration.** Serum (3–5 × dilutions) was added into microtitre plates coated with irradiated virus (2×10^8 p.f.u. equivalent/ml) and incubated for 1 h. The plates were then washed and antibody detected with peroxidase-conjugated rabbit anti-mouse IgG, IgG1 or IgG2a (Dako) and developed with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). The plates were read on a Bio-kinetics Reader (Bio-Tek Instrument) at 405 nm. The end-point was calculated as the highest dilution of serum which gave an absorbance reading twice that of the background (with normal mouse serum).

■ **Flow cytometry analysis.** Spleen cells were pooled from groups of nine or ten mice and analysed for $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ subsets using the appropriate monoclonal antibody (Becton Dickinson). Labelled cells were analysed by flow cytometry (FACScan, Becton Dickinson).

■ **T cell proliferation.** Draining lymph node cells from infected mice were cultured (10^6 cells/ml) in medium (RPMI 1640, Gibco) containing 0·5% FCS and 0·5% normal mouse serum) in flat-bottom 96-well plates (Nunclon) with graded concentrations (5×10^1–5×10^6 p.f.u. equivalent/ml) UV-irradiated HSV-1 for 3–4 days. The cells were then pulsed with [^{3}H]thymidine [1 μCi/well (37 kBq per well), Amersham] for a further 16 h, harvested and the radioactivity counted in a Beta-plate counter (Pharmacia).

■ **Cytokine production and detection.** Spleen cells from infected mice were cultured (2×10^6 cells/ml) in medium (RPMI 1640 and 10% FCS) in flat-bottom 24-well plates with graded concentrations (10^3–10^7 p.f.u. equivalent/ml) of UV-irradiated virus, or with concanavalin A (ConA) (2·5 μg/ml) or LPS (5 μg/ml) for up to 7 days. Cell-free culture supernatants were collected at 1, 2, 3, 4 and 7 days and cytokine concentrations determined by ELISA in 96-well plates (Immulon 4). IL-12 was captured with a combination of rat monoclonal antibodies to mouse IL-12 P40 (C15.1.2 and C15.6, kind gifts of the Genetic Institute, Cambridge, Mass., USA), and detected with a rabbit anti-mouse IL-12 antibody (Rab.74.6, raised in our laboratory). IFN-γ was captured with a rat monoclonal antibody to mouse IFN-γ (R46AT) and detected with a rabbit anti-mouse IFN-γ antibody. IL-4 was captured with a rat monoclonal anti-IL-4 antibody (TRFK4, PharMingen) and detected with a biotinylated rat anti-mouse IL-4 antibody (BVD6-24G2, PharMingen). ELISA was developed with HRP-conjugated donkey anti-rabbit IgG (SAPU) for IL-12 and IFN-γ, or HRP-conjugated StrepAvidin (SAPU) for IL-4, and followed using TMP HRP substrate (Dynatech, UK). The absorbance was read on a multi-scan (MR5000, Dynatech) at 630 nm. Recombinant cytokines were used as reference standards.

■ **Statistical analysis.** Statistical significance ($P < 0·05$) was calculated by the Mann–Whitney test (Minitab software program).

Results and Discussion

iNOS-deficient mice, together with their heterozygous controls, were infected in the footpad with 10-fold dilutions of HSV-1 Glasgow strain 17^+ (10^3–10^6 p.f.u. per mouse). Disease development and mortality were monitored for 42 days post-infection. The LD_{50} of the heterozygotes was 9×10^5 p.f.u. per mouse, which is comparable to that of normal strain BALB/c mice (Robertson *et al.*, 1992). This was reduced to 1×10^4 p.f.u. per mouse in the mutant iNOS-deficient mice. Compared with similarly infected heterozygous control mice, the mutant mice also displayed a significant delay in their ability to clear virus from the footpad and the DRG (Fig. 1*a*, *b*). iNOS-deficient mice that survived HSV-1 infection of 10^4–10^5 p.f.u. per mouse exhibited a marked increase in the frequency of virus reactivation in the DRG compared with heterozygous mice that had recovered from the same dose of virus (Fig. 1*c*, *d*). For ganglia that are not directly supplying the sciatic nerve (T11, T12, L1, L2, S2 and S3) and where virus is spread through the spinal cord, the frequencies of virus reactivation were 85% for the iNOS-deficient mice and 14% for heterozygous control mice.

We next investigated the immune response of acutely infected mice. Heterozygous mice infected with 10^5 p.f.u. of HSV-1 per mouse produced HSV-1-specific antibody detectable 5 days after infection and peaking by day 7. Mutant mice produced significantly higher concentrations of specific antibody from day 5 which progressed through to day 11 (Fig. 2*a*). All the antibody was of the IgG2a isotype; no IgG1 antibody was detected (data not shown). This is consistent with a previous finding that cellular rather than humoral immunity is required for protection against acute HSV infection (Nash & Wildy, 1983; Chan *et al.*, 1985). During the latent phase of infection by sublethal doses of virus, there was no significant difference between mutant and heterozygous mice in their antibody titre 42 days post-infection ($14\,000 \pm 2133$ vs $16\,111 \pm 3093$). Five days after infection with 10^5 p.f.u. of HSV per mouse, draining lymph node cells were collected and cultured with graded doses of UV-irradiated virus *in vitro*. Cells from the heterozygous mice produced a modest but significant proliferative response when cultured with 5×10^3–5×10^4 p.f.u./ml of irradiated virus. This level was markedly elevated in cells from the mutant mice (Fig. 2*b*).

We have previously demonstrated that adoptive transfer of $CD4^+$ T cells primed by glycoprotein B of HSV can protect mice against acute HSV infection (Chan *et al.*, 1985). Therefore, spleen cells from the infected mice were examined for T cell distribution and cultured with irradiated virus, ConA or LPS *in vitro*, followed by determination of the concentations of IFN-γ and IL-4 in the culture supernatants. Flow cytometric analysis showed that 40 days after HSV-1 infection, spleen cells (pooled from nine to ten mice per group) from the iNOS-deficient mice

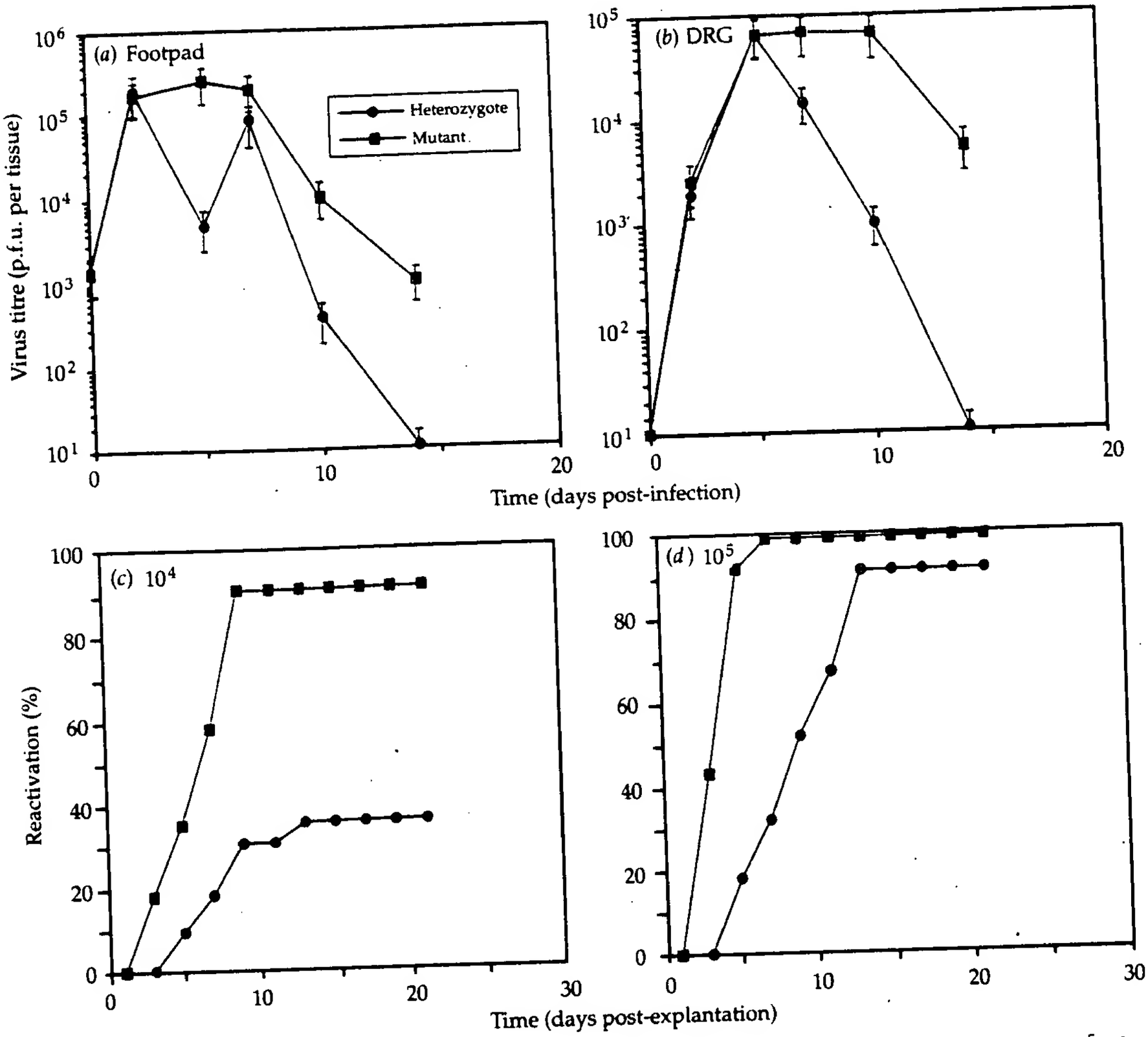


Fig. 1. Virus isolation and reactivation in mice infected with HSV-1. Mice were injected in the right rear footpad with 10^5 p.f.u. of virus per mouse and sacrificed at regular intervals. Virus titres in the infected footpad (*a*) and the DRG (*b*) were determined by titration in BHK21/C13 cells. Each point represents the mean virus yield in p.f.u. per tissue sample of two to three mice. Standard errors are shown as vertical bars. Typically, in this type of experiment, due to physical losses, a 100× decline in titre immediately post-infection is observed. Thus the actual virus titre is probably 100× higher than shown. The pattern seen in the heterozygous mice is typical of other normal strains of mouse where virus titre in the footpad peaks on day 2 and declines thereafter, followed by a second peak on day 7 post-infection. No virus was detected by day 14. In the DRG, virus was first detected on day 2, when virus would have travelled up the sciatic nerve, peaked on day 5 following replication in the DRG and thereafter rapidly declined, with no virus detectable by day 14, when a latent infection would have been established. The second peak in the footpad is believed to be due to virus travelling back down the sciatic nerve following replication in the DRG. The time-course of explant reactivation in mice infected with 10^4 (*c*) or 10^5 (*d*) p.f.u. of virus per mouse was also examined. Six weeks after inoculation of 4-week-old mice in the right rear footpad, ten DRG were explanted from the right side of surviving mice (four to five mice per group) and assayed every second day for the release of infectious virus in BHK21/C13 cells. The percentage of total DRG reactivating at each time point is shown. Similar results were obtained in two additional experiments.

contained more $CD3^+$ cells (37·9% vs 31·2%, $P < 0·05$) and $CD3^+CD4^+$ cells (28·7% vs 20·91%, $P < 0·05$) but similar fractions of $CD3^+$ $CD8^+$ cells (7·9% vs 6·98%) compared with heterozygous mice. Cells from infected iNOS-deficient mutant mice produced significantly more IFN-γ and less IL-4 than those from similarly infected heterozygous control mice (Fig. 3), indicating a preferential expansion of Th1 cells in the absence of iNOS. These results are consistent with the observation that NO inhibits the development of Th1 cells (Wei *et al.*, 1995; Taylor-Robinson *et al.*, 1994). Cells from mutant mice produced significantly more IL-12 than cells from heterozygous mice at all time points tested (Fig. 4). Since IL-12 is a major inducer of Th1 cell development (Trinchieri, 1993) and is predominantly produced by macrophages, these data indicate that NO produced endogenously by iNOS in macrophages could inhibit the production of IL-12, thereby limiting the development of Th1 cells. The mechanism by which NO inhibits IL-12 synthesis is at present unclear.

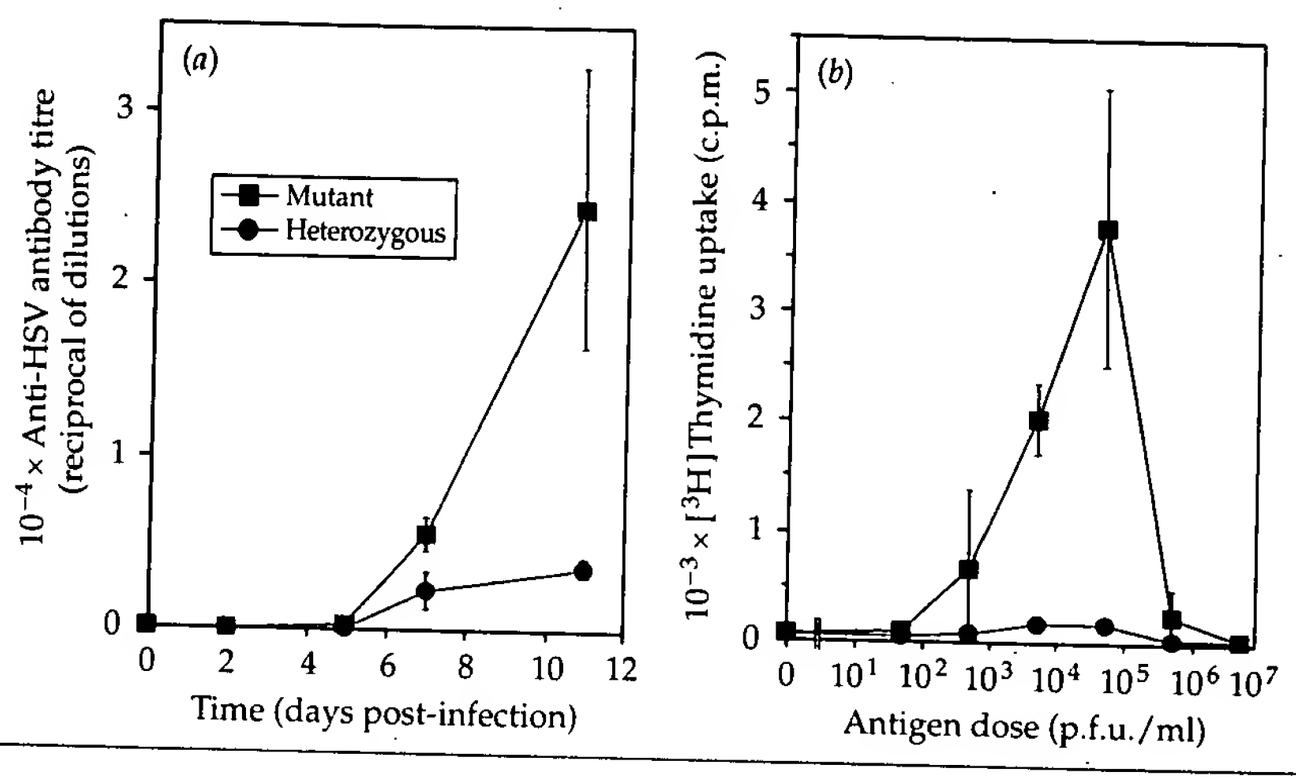


Fig. 2. (*a*) Virus-specific antibody response. Mice were infected as described in the legend to Fig. 1. Sera were collected when mice were sacrificed, and titrated for anti-HSV antibody by ELISA. Each point represents the mean of triplicate wells ± 1 SD from two to three mice. (*b*) Proliferative response of draining lymph node cells from mice infected 5 days previously with 10^5 p.f.u. of HSV-1 per mouse. For details see Methods. Each point represents the mean of triplicate cultures ± 1 SD from a pool of cells from three mice. Similar results were obtained in two additional experiments.

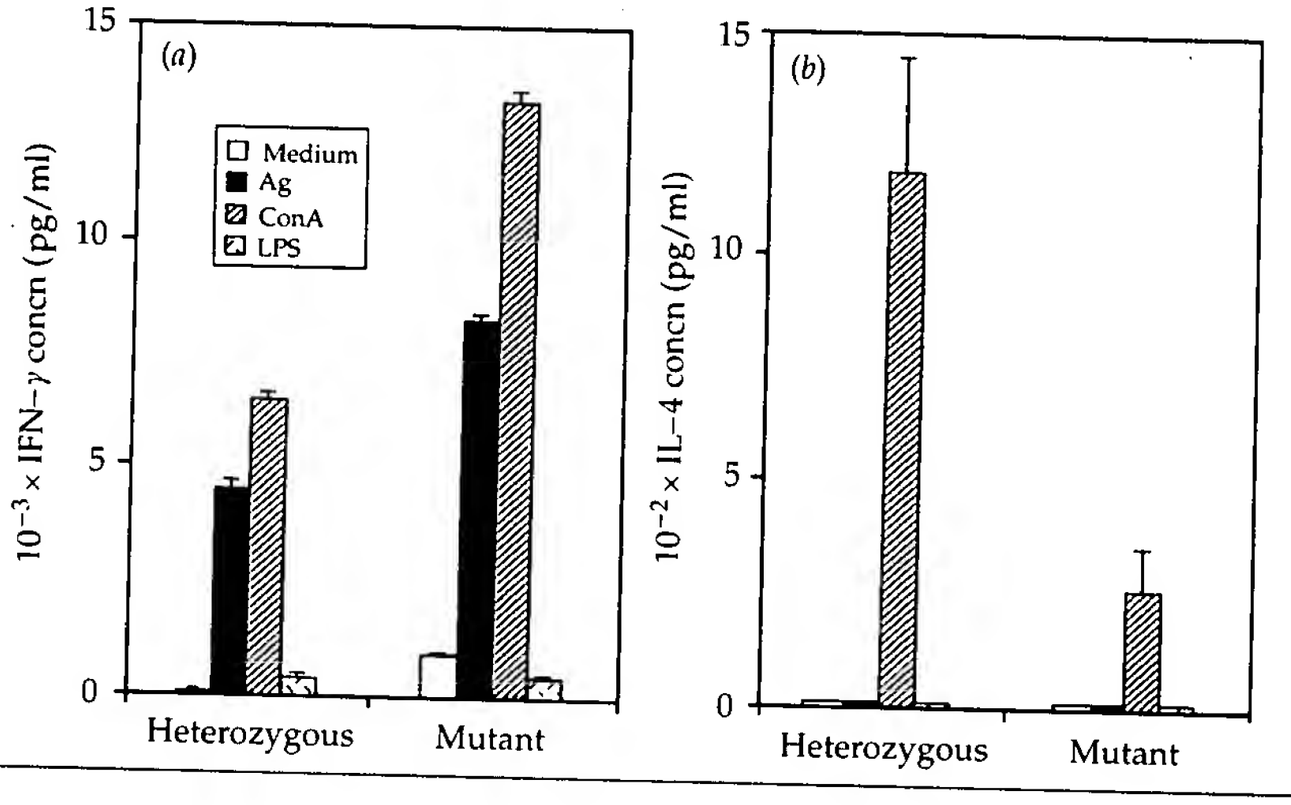


Fig. 3. IFN-γ (*a*) and IL-4 (*b*) produced by spleen cells from mice infected 40 days previously with 10^4 p.f.u. of HSV-1 per mouse and cultured *in vitro* with optimal dose of antigen (10^6 p.f.u. equivalent/ml of irradiated HSV-1) for 4 days, or with ConA (2·5 μg/ml) or LPS (5 μg/ml) for 3 days. Data for peak time points are shown. Culture supernatants were titrated for the cytokines by ELISA. Data shown are means ± 1 SD of triplicate cultures. Similar results were obtained in two additional experiments.

However, it is unlikely to be the consequence of higher virus load, since IL-12 is induced rapidly after infection (Kanangat *et al.*, 1996; Scott, 1993; Ma *et al.*, 1996) and the virus replication rate was similar in both iNOS-deficient and control mice for the first 2 days after infection (Fig. 1*a*, *b*). Furthermore, a similar phenomenon was observed for *Leishmania major* infection (unpublished).

Using inhibitors for NO synthase, NO has been shown to inhibit the *in vitro* replication of ectromelia virus, HSV-1, vaccinia virus, vesicular stomatitis virus and human immunodeficiency virus (Croen, 1993; Karupiah *et al.*, 1993; Burkrinsky *et al.*, 1995; Mannick *et al.*, 1994; Bi & Reiss, 1995). These inhibitors have also been shown to significantly exacerbate ectromelia virus infection (Karupiah *et al.*, 1993), HSV-1-induced pneumonia (Adler *et al.*, 1997) and influenza virus-induced pneumonia (Akaike *et al.*, 1996). However, treatment of mice with NOS inhibitors failed to influence the course of vaccina virus (Rolph *et al.*, 1996), influenza virus (J. P. Tite & F. Y. Liew, unpublished), or lymphocytic choriomeningitis virus (R. M. Zinkernagel & F. Y. Liew, unpublished) infections. Furthermore, NO appears to protect mice from fatal encephalitis induced by Sindbis virus by a mechanism that does not directly involve the immune response or inhibition of virus growth (Tucker *et al.*, 1996). The present study provides direct *in vivo* evidence that iNOS plays a role in inhibiting HSV-1 replication and in host protection. The mechanism by which NO limits virus replication is at present unclear. It is likely that NO acts as a direct effector molecule rather than indirectly through the enhancement of host immune responses because iNOS-deficient mice had reduced resistance to HSV-1 infection in spite of enhanced Th1 response, which is known to be host-protective against this virus. NO could restrict virus replication by lysing target cells or by direct damage to viral particles as it is generally accepted that macrophage activation is important in determining the outcome of HSV infection, and that resistance correlates with the ability of macrophages to restrict HSV replication and dissemination (Johnson, 1964). This mechanism predicts that NO is important in infections with macrophages as target cells, but is unlikely to have a major role against virus infections in which $CD8^+$ killer cells are the main effector mechanism. iNOS-deficient mice contain similar levels of $CD8^+$ T cells as heterozygous mice following HSV-1

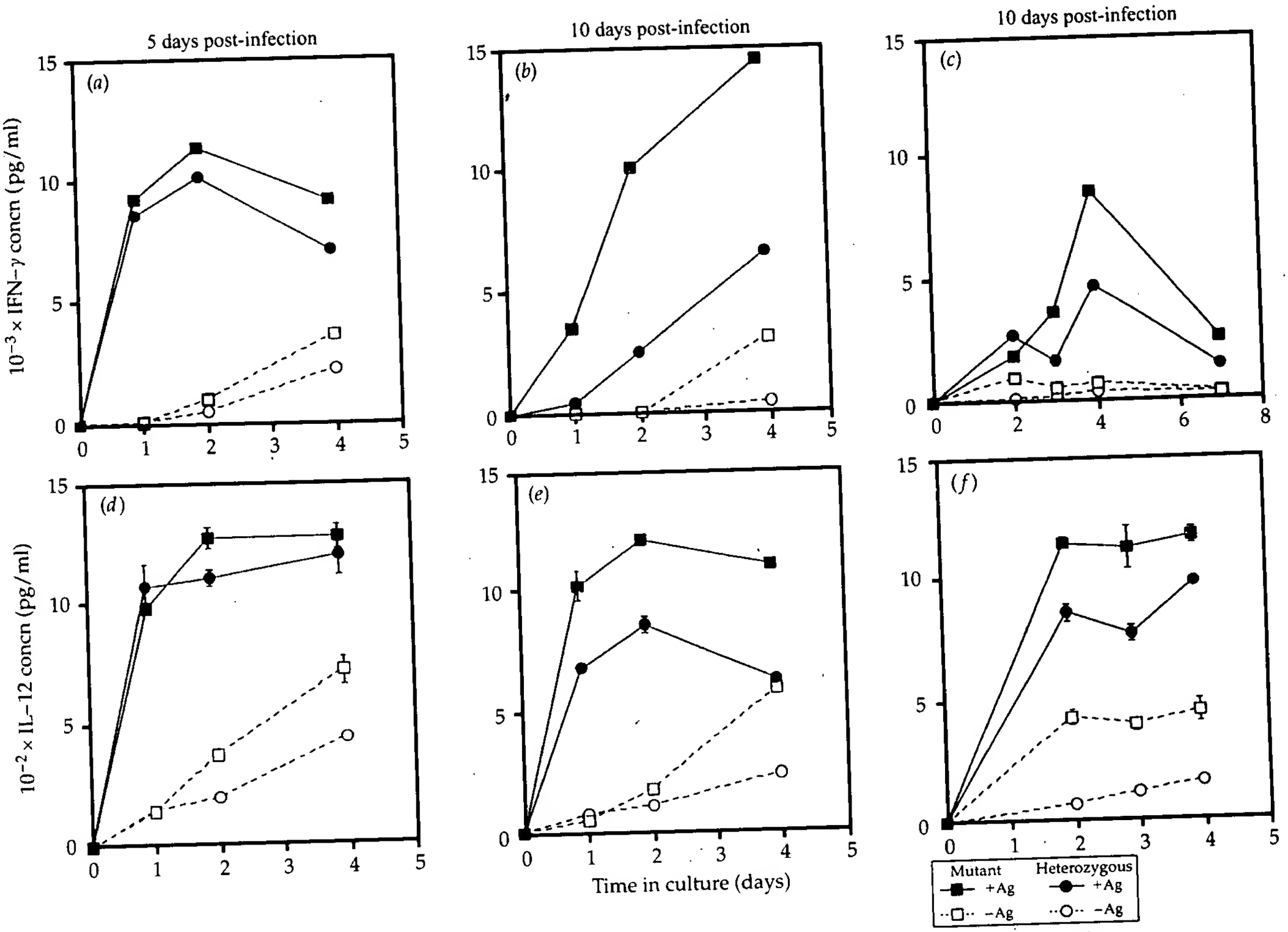


Fig. 4. IFN-γ (*a*, *b*, *c*) and IL-12 (*d*, *e*, *f*) produced by pooled spleen cells from mice infected at 5 and 10 days (10^5 p.f.u. per mouse) or 40 days (10^4 p.f.u. per mouse) with HSV-1. Cells were cultured with or without UV-irradiated virus for the periods indicated. Data for optimal cytokine production are shown. Optimal concentrations of antigen for cytokine production were 10^7 p.f.u./ml for cells from day 5 post-infection, and 10^6 p.f.u./ml for cells from 10 and 40 days post-infection. Cytokine concentrations were determined by ELISA. Each point represents the mean ±1 SD, $n = 3$. Some of the error bars are obscured by the symbols. Similar results were obtained in two additional experiments.

infection. The ability of iNOS-deficient mice to control low doses of infection indicates that mechanisms in addition to NO are involved in the resistance against HSV-1 infection. Furthermore, macrophages are not the only cell type HSV-1 infects.

A major finding in this report is the preferential induction of Th1 cells in mice deficient in iNOS. This is likely to result from inhibition of IL-12 synthesis by NO. It is now generally agreed that the balance between Th1 (producing IFN-γ) and Th2 (producing IL-4) subsets of $CD4^+$ T cells determines the outcome of many infectious and autoimmune diseases (Sher & Coffman, 1992; Liew, 1992; Mason & Fowell, 1992). Our data suggest that iNOS is important in maintaining a state of immunological balance, preventing the overexpansion of Th1 cells which have been implicated in a range of immunopathologies. They also suggest that iNOS inhibitors may be useful adjuvants for vaccination where an enhanced Th1 cell response is essential for protective immunity.

We thank the Medical Research Council of Great Britain, the Joint Research Board of the Barts NHS Trust and the Wellcome Trust for financial support.

References

Adler, H., Beland, J. L., Del-Pan, N. C., Kobzik, L., Brewer, J. P., Martin, T. R. & Rimm, I. J. (1997). Suppression of herpes simplex virus type I (HSV-1)-induced pneumonia in mice by inhibition of inducible nitric oxide synthase (iNOS, NOS2). *Journal of Experimental Medicine* **185**, 1533–1540.

Akaike, T., Noguchi, Y., Ijiri, S., Setoguchi, K., Suga, M., Zheng, Y. M., Dietzschold, B. & Maeda, H. (1996). Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. *Proceedings of the National Academy of Sciences, USA* **93**, 2448–2453.

Albina, J. E., Abate, J. A. & Henry, W. L., Jr (1991). Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation: role of IFN-γ in the induction of the nitric oxide-synthesizing pathway. *Journal of Immunology* **147**, 144–148.

Bi, Z. & Reiss, C. S. (1995). Inhibition of vesicular stomatitis virus infection by nitric oxide. *Journal of Virology* **69**, 2208–2213.

Burkrinsky, M. I., Nottet, H. S. L. M., Schmidtmayerova, N., Dubrovsky, L., Mullins, M. E., Lipton, S. A. & Gendelman, H. E. (1995). Regulation of nitric oxide activity in HIV-infected monocytes: implication for HIV-associated neurological disease. *Journal of Experimental Medicine* **181**, 735–745.

Chan, W. L., Lukic, M. L. & Liew, F. Y. (1985). Helper T cells induced by an immunopurified herpes simplex virus type I (HSV-1) 115 kilodalton glycoprotein (gB) protect mice against HSV-1 infection. *Journal of Experimental Medicine* **162**, 1304–1318.

Croen, K. D. (1993). Evidence for an antiviral effect of nitric oxide. *Journal of Clinical Investigation* **91**, 2446–2452.

Johnson, R. T. (1964). The pathogenesis of herpes virus encephalitis. II. A cellular basis for the development of resistance with age. *Journal of Experimental Medicine* **120**, 359–369.

Kanangat, S., Thomas, J., Gangappa, S., Babu, J. S. & Rouse, B. T. (1996). Herpes simplex virus type 1-mediated up-regulation of IL-12 (p40) mRNA expression. *Journal of Immunology* **156**, 1110–1116.

Karupiah, G., Xie, Q.-w., Buller, R. M. L., Nathan, C., Duarte, C. & MacMicking, J. D. (1993). Inhibition of viral replication by interferon-γ-induced nitric oxide synthase. *Science* **10**, 1445–1448.

Liew, F. Y. (1992). Induction, regulation and function of T-cell subsets in leishmaniasis. *Chemical Immunology* **54**, 117–135.

Liew, F. Y. (1995). Interactions between cytokines and nitric oxide. *Advances in Neuroimmunology* **5**, 201–209.

Ma, X., Chow, J. M., Gri, G., Carra, G., Gerosa, F., Wolf, S. F., Dzialo, R. & Trinchieri, G. (1996). The interleukin 12 p40 gene promoter is primed by interferon γ in monocytic cells. *Journal of Experimental Medicine* **183**, 147–157.

MacLean, A. R., Ul-Fareed, M., Robertson, L., Harland, J. & Brown, S. M. (1991). Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence-related sequences in Glasgow strain 17^+ between immediate early gene 1 and the 'a' sequence. *Journal of General Virology* **72**, 631–639.

MacPherson, I. & Stoker, M. G. (1962). Polyoma transformation of hamster cell lines: an investigation of genetic factors affecting cell competence. *Virology* **16**, 147–151.

Mannick, J. B., Asano, K., Izumi, K., Kieff, E. & Stamler, J. S. (1994). Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein–Barr virus reactivation. *Cell* **79**, 1137–1146.

Mason, D. & Fowell, D. (1992). T-cell subsets in autoimmunity. *Current Opinion in Immunology* **4**, 728–732.

Moncada, S. & Higgs, A. (1993). The L-arginine–nitric oxide pathway. *New England Journal of Medicine* **329**, 2002–2012.

Nash, A. A. & Wildy, P. (1983). Immunity in relation to the pathogenesis of herpes simplex virus. In *Human Immunity to Viruses*, pp. 179–192. Edited by F. A. Ennis. New York: Academic Press.

Nathan, C. & Xie, S. Q. (1994). Regulation of biosynthesis of nitric oxide. *Journal of Biological Chemistry* **269**, 13725–13728.

Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty percent endpoints. *American Journal of Hygiene* **27**, 493–449.

Robertson, L. M., MacLean, A. R. & Brown, S. M. (1992). Peripheral replication and latency reactivation kinetics of the non-neurovirulent herpes simplex virus type 1 variant 1716. *Journal of General Virology* **73**, 967–970.

Rolph, M. S., Ramshaw, I. A., Rockett, K. A., Ruby, J. & Cowden, W. B. (1996). Nitric oxide production is increased during murine vaccinia virus infection, but may not be essential for virus clearance. *Virology* **217**, 470–477.

Scott, P. (1993). IL-12: the initiation cytokine for cell-mediated immunity. *Science* **260**, 696.

Sher, A. & Coffman, R. L. (1992). Regulation of immunity to parasites by T cells and T-cell derived cytokines. *Annual Review of Immunology* **10**, 385–409.

Taylor-Robinson, A. W., Liew, F. Y., Severn, A., Xu, D., McSorley, S. J., Garside, P., Padron, J. & Phillips, R. S. (1994). Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells. *European Journal of Immunology* **24**, 980–984.

Trinchieri, G. (1993). Interleukin-12 and its role in the generation of Th1 cells. *Immunology Today* **14**, 335–338.

Tucker, P. C., Griffin, D. E., Hchoi, S., Bui, N. & Wesselingh, S. (1996). Inhibition of nitric oxide synthesis increases mortality in sindbis virus encephalitis. *Journal of Virology* **70**, 3972–3977.

Wei, X.-Q., Charles, I. G., Smith, A., Ure, J., Feng, G.-J., Huang, F.-P., Xu, D. M., Muller, W., Moncada, S. & Liew, F. Y. (1995). Altered immune response in mice lacking inducible nitric oxide synthase. *Nature* **375**, 408–411.

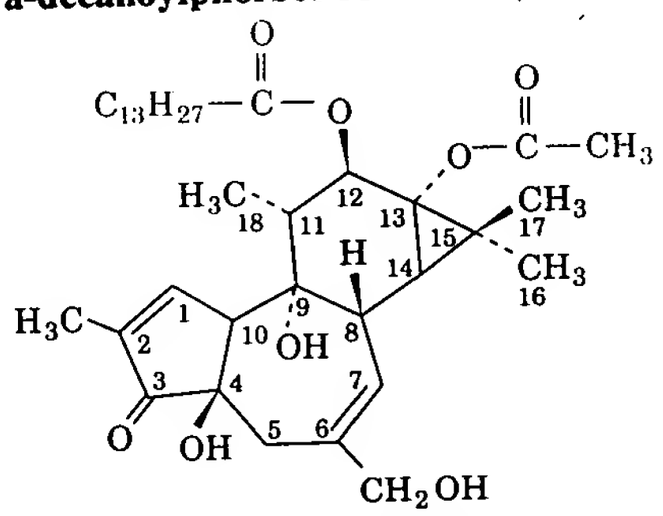
Received 4 September 1997; Accepted 11 November 1997

$G_\beta G_\gamma$ functions to activate certain PLC isoforms, notably **PLC-β2.**

In contrast to G-protein-linked receptors, certain ligand-activated and thus autophosphorylated RTKs, including those for PDGF and EGF (Fig. 34-103), directly activate **PLC-γ1** by binding to this isoform via its two SH2 domains (it also has an SH3 domain) and phosphorylating at least three of its Tyr residues. PLC-γ1's association with the membrane-bound RTK, besides facilitating its phosphorylation, brings this otherwise cytosolic enzyme into contact with its substrate, PIP_3, in the inner leaflet of the plasma membrane. In *T* cells, PLC-γ1 is likewise phosphorylated through the action of activated *T* cell receptors, but here these tyrosine kinase–associated receptors recruit members of the Src family such as Fyn and Lck to carry out the phosphorylation.

IP_3 and DG are rapidly recycled to form PIP_2 through the bicyclic metabolic pathway diagrammed in Fig. 34-109. Some of these inositol phosphates, as well as many not appearing in Fig. 34-109, also act as signal molecules in certain cells, thereby increasing an organism's ability to respond to complex stimuli. Intriguingly, the enzyme that catalyzes the hydrolysis of **inositol-1-phosphate (IP_1), IP_1 phosphatase,** is inhibited by Li^+. The therapeutic efficacy of Li^+ in controlling the incapacitating mood swings of manic-depressive individuals therefore suggests that this mental illness is caused by an aberration in a phosphoinositide signaling system in the brain, possibly causing abnormal activation of Ca^{2+}-mobilizing receptors.

The activating effects of IP_3 and DG explain many cellular phenomena. In skeletal muscle, for example, IP_3 mobilizes Ca^{2+} from the sarcoplasmic reticulum. Since Ca^{2+} triggers muscle contraction (Section 34-3C), this observation suggests that nerve impulses mobilize Ca^{2+} by releasing neurotransmitters in the myofibril's T tubules (Fig. 34-67), which then bind to PLC-activating receptors. In a second example, several polypeptide growth factors, including PDGF, act to mobilize IP_3 and DG, which in turn stimulate cell proliferation. The v-*sis* oncogene, which it will be recalled specifies an analog of PDGF (Section 33-4C), may therefore act to permanently switch on PIP_2 degradation, thus forcing the cell into a state of continuous proliferation. Several other oncogene products, including v-Src, are thought to aberrantly activate the synthesis of PIP_2 from its precursors (Fig. 34-109). Similarly, **phorbol esters** such as **12-*O*-tetra-decanoylphorbol-13-acetate,**

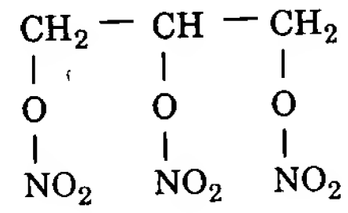


12-*O*-Tetradecanoylphorbol-13-acetate

which are potent activators of protein kinase C (they structurally resemble DG), are the most effective known **tumor promotors** (substances that are not in themselves carcinogenic but increase the potency of known carcinogens; phorbol esters induce the synthesis of the transcription factor AP-1, the product of the *c-jun* proto-oncogene; Section 33-4C). Clearly, the phosphoinositide cascade plays a central role in the control of cellular metabolism.

NO and Possibly CO Are Biological Messengers

Nitric oxide (NO) is a reactive and toxic free radical gas. Thus, it came as a great surprise that *this molecule functions as an intercellular signal in regulating blood vessel dilation and serves as a neurotransmitter. It also functions in the immune response.* The role of NO in vasodilation was discovered through the observation that substances such as acetylcholine (Section 34-4C) and bradykinin (Section 6-4B), which act through the phosphoinositide signaling system to increase the flow through blood vessels by eliciting smooth muscle relaxation, require an intact endothelium overlying the smooth muscle. Evidently, endothelial cells respond to the presence of these vasodilation agents by releasing a diffusible and highly labile substance (half-life ~5 s) that induces the relaxation of smooth muscle cells. This substance was identified as NO, in part, through parallel studies identifying NO as the active metabolite that mediates the well-known vasodilating effects of antianginal organic nitrates such as **nitroglycerin**



Nitroglycerin

(**angina pectoris** is a disease caused by insufficient blood flow to the heart muscle, leading to severe chest pain).

NO is synthesized by **NO synthase (NOS),** which catalyzes the NADPH-dependent reaction of L-arginine with O_2 to yield NO and the amino acid citrulline (Fig. 34-110). NOS is a homodimeric protein of 125- to 160-kD subunits that are homologous to **cytochrome P_{450} reductase,** an enzyme involved in detoxification processes. Each NOS subunit contains one FMN, one FAD, one tetrahydobiopterin (Fig. 24-21), and one Fe(III)-heme, cofactors that presumably facilitate the 5-electron oxidation of L-arginine to yield NO. The enzyme is activated by Ca^{2+} through its interaction with Ca^{2+}–calmodulin. Hence, the stimulatory action of vasodilatants on the phosphoinositide signaling system in endothelial cells to produce an influx of Ca^{2+} results in the synthesis of NO.

NO rapidly diffuses across cell membranes, although its high reactivity prevents it from getting >1 mm from its site of synthesis (in particular, it efficiently reacts with both oxyhemoglobin and deoxyhemoglobin: $NO + HbO_2 \rightarrow NO_3^- + Hb$; and $NO + Hb \rightarrow HbNO$). *The physiological target of NO in smooth muscle cells is* ***guanylate cyclase,***

QR1.A47 A35

STIC-ILL

+
MIC

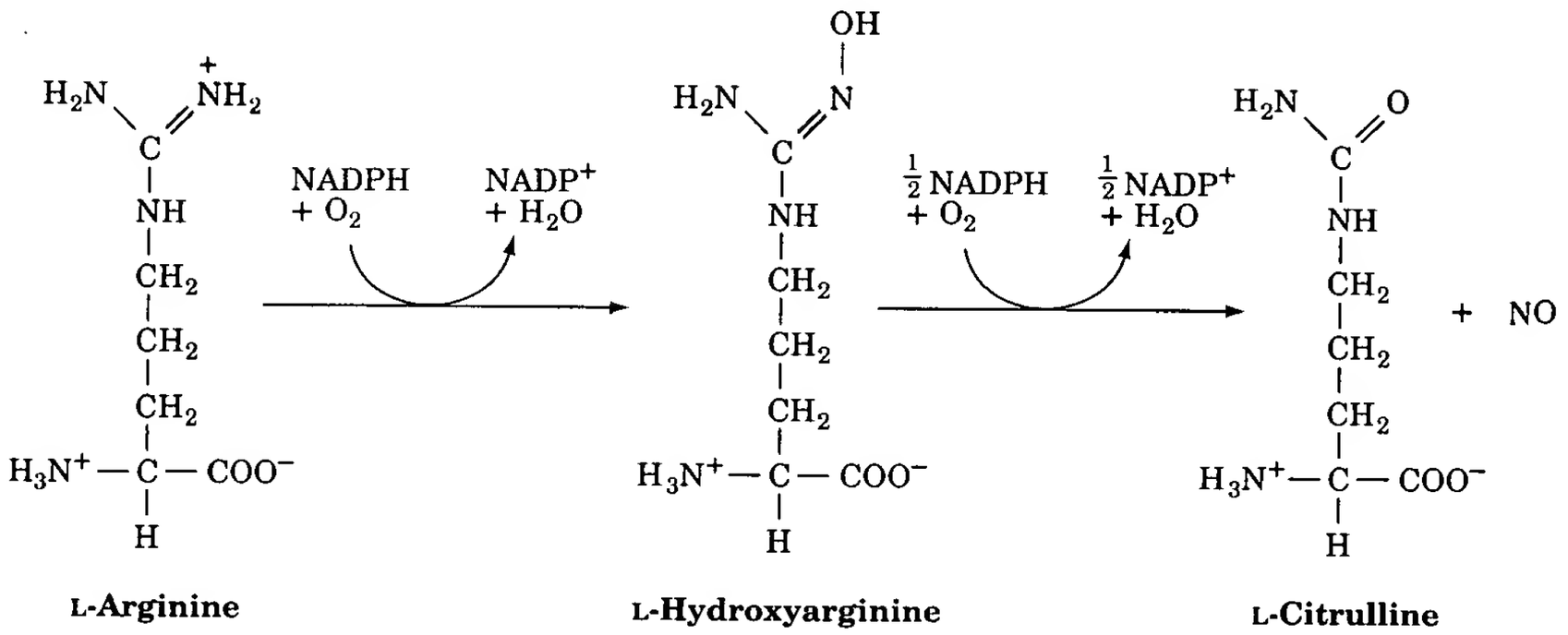
From: Prasad, Sarada
Sent: Tuesday, March 06, 2001 5:33 PM
To: STIC-ILL

Please fetch me the following full text citations for the case # 09,395,038

1. Philosophical transactions of the roayal Society of London B Biological Sciences (Sept. 29, 1997) vol. 352, No. 1359, pages 1311-1315.(cytokines and nitric oxide as effector molecules against parasitic infections.
2. J Immunol. 1994, vol.153, No.11, pages 5200-5209. (Elevated expression of Th1 cytokines and nitric oxide synthase in the lungs of vaccinated mice after challenge infection with schistosoma mansoni.)
3. Cancer immunol, Immunotherpy, 1996 jan, vol.42, No.1, pages 38-46. (Effects of N-methyl arg, an inhibitor of nitric oxide synthesis on IL-2 induced capillary leakage and anti-tumor responses innhealthy and tumor bearing mice.
4. Infection and immunity 1997, vol. 65, No. 4 1307-1312. (Expression of cytokines and inducible nitric oxuide synthase mRNA in the lungs of mice infected with cryptococcus neoformans: effects of interleukin 12).
5. Journal of general virology 1998, vol.79, No. 4, pages 825-830 (Mice lacking inducible niric oxide synthase are more susceptible to herpers simplex virus infection despite enhanced Th1 cells responses)
6. J Neuroimmunology 1996, vol.68, No. 1-2, pages 101-108. (Interferon gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons.)
7. J Experimental medicine 1996, vol.183, No. 4, pages 1447-1459. (The role of interleukin 12 and nitric oxide in the development of spontaneous auroimmune disese in mrl...mice).

thank you,

Sarada Prasad, Ph.D.
Patent Examiner
Art Unit 1646
Room CM1-8B17
Tel: 703-305-1009



L-Arginine **L-Hydroxyarginine** **L-Citrulline**

FIGURE 34-110. The NO synthase (NOS) reaction. The L-hydroxyarginine intermediate is tightly bound to the enzyme.

which catalyzes the reaction of GTP to yield **3′,5′-cyclic GMP (cGMP),** an intracellular second messenger that resembles cAMP. cGMP causes smooth muscle relaxation through its stimulation of protein phosphorylation by **cGMP-dependent protein kinase.** NO reacts with guanylate cyclase's heme prosthetic group to yield **nitrosoheme,** whose presence increases the enzyme's activity 50-fold, presumably via a conformation change resembling that in hemoglobin upon binding O_2 (Section 9-2B). Thus, *NO functions to transduce hormonally induced increases in intracellular [Ca^{2+}] in endothelial cells to increased rates of production of cGMP in neighboring smooth muscle cells.*

NO also mediates vasodilation through endothelium-independent neural stimulation of smooth muscle. In this signal transduction pathway, which is responsible for the dilation of cerebral and other arteries as well as penile erection, nerve impulses cause an increased [Ca^{2+}] in nerve terminals, thereby stimulating neuronal NOS (which is ~55% homologous to endothelial NOS). The resultant NO diffuses to nearby smooth muscle cells, where it binds to guanylate cyclase and activates it to synthesize cGMP as described above.

A third type of NOS, which is calmodulin- independent, is transcriptionally induced in macrophages and **neutrophils** (white blood cells that function to ingest and kill bacteria), as well as in endothial and smooth muscle cells. Several hours after exposure to cytokines and/or endotoxins (bacterial cell wall lipopolysaccharides that elicit inflammatory responses; Section 34-2F), these cells begin to produce large quantities of NO and continue doing so for many hours. Activated macrophages and neutrophils also produce superoxide radical ($O_2^-\cdot$), which chemically combines with NO to form the even more toxic **peroxynitrite ($OONO^-$,** which rapidly decomposes to the highly reactive **hydroxide radical, $OH\cdot$,** and NO_2) that they use to kill ingested bacteria. Indeed, NOS inhibitors block the cytotoxic actions of macrophages. In endothelial and smooth muscle cells, cytokines and endotoxins induce a long-lasting and profound vasodilation and a poor response to vasoconstrictors such as epinephrine. The sustained release of NO has been implicated in **endotoxic shock** (an often fatal immune system overreaction to bacterial infection), inflammation-related tissue damage, and in the damage to neurons in the vicinity of but not directly killed by a stroke (which often does greater harm than the stroke itself).

The physical resemblance of CO and NO (both are highly toxic diatomic gases) and the ability of CO to activate guanylate cyclase (presumably by binding to its heme group as does NO) suggests that CO may also act as an intercellular messenger. CO is physiologically generated through the oxidative breakdown of heme to form biliverdin, Fe^{3+}, and CO in a reaction catalyzed by **heme oxygenase (HO;** Section 24-4A). Two forms of HO are known: **HO-1,** which is abundant in spleen and other tissues involved in red cell breakdown; and **HO-2,** which is prevalent in brain tissue. Intriguingly, cytochrome P_{450} reductase, the only mammalian protein known to be homologous to NOS, is the electron donor in the HO reaction. *In situ* hybridization studies indicate that HO-2 mRNA is present in discrete neuronal locations throughout the brain that closely overlap those for guanylate cyclase mRNA but differ somewhat from those of NOS mRNA. The presence of **zinc protoporphyrin IX** (the Zn^{2+} analog of heme), a potent selective inhibitor of HO, results in a large decrease in the concentration of endogenous cGMP. All of these observations constitute strong circumstantial evidence that CO is, in fact, a neurotransmitter.

C. Neurotransmission

In higher animals, the most rapid and complex intercellular communications are mediated by nerve impulses. Neurons (nerve cells; e.g., Fig. 1-10*d*) electrically transmit these signals along their highly extended lengths (commonly over 1 m in larger animals) as traveling waves of ionic currents. Signal transmission between neurons as well as between neurons and muscles or glands, is usually chemically mediated by neurotransmitters. In the remainder of this sec-

INFECTION AND IMMUNITY, Apr. 1997, p. 1307–1312
0019-9567/97/$04.00+0

Vol. 65, No. 4

Expression of Cytokines and Inducible Nitric Oxide Synthase mRNA in the Lungs of Mice Infected with *Cryptococcus neoformans*: Effects of Interleukin-12

KAZUYOSHI KAWAKAMI,* MASAKI TOHYAMA, XIE QIFENG, AND ATSUSHI SAITO

First Department of Internal Medicine, Faculty of Medicine, University of the Ryukyus, 903-01 Okinawa, Japan

Received 2 July 1996/Returned for modification 19 August 1996/Accepted 16 January 1997

We have recently established a murine model of pulmonary and disseminated infection with a highly virulent strain of *Cryptococcus neoformans* and demonstrated that administration of interleukin-12 (IL-12) protected the animals against infection. In this study, we extended these studies by investigating the host defense mechanisms. In particular, we examined the expression of mRNA for helper T-cell 1 (Th1) cytokines (IL-2, lymphotoxin, and gamma interferon [IFN-γ]), Th2 cytokines (IL-4, -6, and -10), macrophage-derived cytokines (tumor necrosis factor alpha [TNF-α], IL-1β, transforming growth factor β [TGF-β], IL-12p40, and IFN-γ-inducing factor [IGIF]), and inducible nitric oxide synthase (iNOS) in the lungs on days 1, 3, 7, and 14 after infection and following treatment with IL-12. There was little or no expression of mRNAs for Th1 cytokines, TNF-α, IL-12p40, IGIF, and iNOS in the infected mice, but expression increased markedly after treatment with IL-12. In contrast, the mRNAs for Th2 cytokines, IL-1β, and TGF-β were detected at considerable levels during the early stages of infection, and, interestingly, expression was not suppressed by IL-12 but rather augmented, particularly during the late stage. Similar results were also obtained for IFN-γ, IL-4, IL-10, and TNF-α measured in the lung homogenates by enzyme-linked immunosorbent assay. These results suggest that the predominance of expression of Th2 cytokines and TGF-β over Th1 cytokines, TNF-α, IL-12p40, IGIF, and iNOS is associated with severe lethal infection in mice and that administration of IL-12 protects infected animals by stimulating Th1 cytokines.

The helper T-cell (Th)-derived cytokines are categorized into two groups. The first comprises Th1 cytokines, including interleukin-2 (IL-2), gamma interferon (IFN-γ), and lymphotoxin (LT). The second group comprises Th2 cytokines, including IL-4, -5, -6, -9, -10, and -13 (31). These groups are known to inhibit the biological activities of each other by producing IFN-γ and IL-4 (31). The Th1 cytokines are strongly involved in the enhancement of cell-mediated immunity (31) and play a central role in the host defense mechanisms against various pathogenic microorganisms (40). IL-12 is produced by macrophages and B cells and stimulates natural killer (NK) cells and T cells to produce IFN-γ (46). More importantly, IL-12 plays a critical role in the development of Th1 cells from naive T cells (17). Recently, another IFN-γ-inducing cytokine, termed the IFN-γ-inducing factor (IGIF), with biological activities similar to IL-12 has been identified and is proposed to be called IL-18 (34, 48). On the other hand, the Th2 cytokines inhibit the production and biological activities of Th1 cytokines (31), thus attenuating host defense mechanisms against pathogenic organisms, as previously shown by increased susceptibility of mice to leishmaniasis by immunological manipulations that enhance the dominance of Th2 cells over Th1 cells (2, 25). Thus, the commitment of specific Th cells to differentiation into Th1 or Th2 cells may determine the host susceptibility to particular pathogenic microorganisms.

Cryptococcus neoformans, a ubiquitous fungal microorganism, has attracted attention because it causes a life-threatening infection in patients with impaired cell-mediated immunity, such as AIDS (44). In infections caused by this pathogen, cellular immunity, mediated mostly by $CD4^+$ T cells and macrophages, forms a central role in host defense (16, 18, 29). Recent studies from our group as well as other laboratories have demonstrated that IFN-γ is important in protecting mice against this infection through induction of macrophage anticryptococcal activity (22, 37). In mice, such activity is mediated to a large extent by L-arginine-dependent killing mechanisms (1, 45).

Recently, we established a murine model of pulmonary and disseminated cryptococcosis and demonstrated that IL-12 protected mice from lethal infection with *C. neoformans* by inducing production of IFN-γ (23). However, the mechanism for the IL-12 effect is not fully understood. In this study, we examined the kinetics involved in generation of Th1- and Th2-associated cytokines, macrophage-derived cytokines, and inducible nitric oxide synthase (iNOS) in an effort to characterize host defense mechanisms against cryptococcal infection. We also examined the effects of IL-12 treatment on the synthesis of these cytokines and iNOS.

MATERIALS AND METHODS

Animals. Female (BALB/c × DBA/2)F_1 mice were purchased from SLC Japan (Hamamatsu, Japan) and used at the age of 7 to 10 weeks. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of our university. The anticryptococcal activity of macrophages derived from this strain of mice is not detected in the absence of stimulation with IFN-γ plus lipopolysaccharide, while it is markedly induced after stimulation (3). All mice were housed in a pathogen-free environment and received sterilized food and water at the Laboratory Animal Center for Biomedical Science in University of the Ryukyus.

***C. neoformans*.** A serotype A encapsulated strain of *C. neoformans*, YC-11, was obtained from a patient with pulmonary cryptococcosis. The strain showed thick capsule (7.6 ± 2.3 μm; $n = 59$) when examined shortly after harvesting from the infected lungs (not shown). The yeast cells were cultured on potato dextrose agar plates for 3 to 4 days before use.

Intratracheal instillation of microorganisms. Mice were anesthetized by an intraperitoneal injection of 70 mg of pentobarbital (Abbott Laboratories, North

* Corresponding author. Mailing address: First Department of Internal Medicine, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-01, Japan. Phone: 81(98)895-3331, ext. 2438. Fax: 81(98)895-3086.

Chicago, Ill.) per kg of body weight and restrained on a small board. Live *C. neoformans* cells (10^5) were inoculated in a volume of 50 μl per mouse by inserting a blunted 25-gauge needle into and parallel to the trachea.

IL-12. Recombinant murine IL-12 (specific activity, 2.5×10^8 Roche units/mg of protein) was kindly provided by Hoffmann-La Roche Inc. (Nutley, N.J.). IL-12 was intraperitonealy administered at a dose of 0.1 μg per mouse daily for 7 days from the day of infection.

Extraction of RNA and reverse transcription-PCR (RT-PCR). Total RNA was extracted from the lungs of mice at various time points after instillation of *C. neoformans* by the acid guanidinium thiocyanate-phenol-chloroform method as described by Chomczynski and Sacchi (6). For this purpose, 30 to 70 μg of RNA was obtained from one set of lungs and resuspended in 50 μl of diethylpyrocarbonate-treated distilled water.

Subsequently, reverse transcription was carried out by mixing 5 μg of sample RNA solution (15 μl) with 2 μl of hexadeoxyribonucleotide mixture (GIBCO BRL, Life Technologies, Tokyo, Japan). This solution was incubated for 2 min at 95°C and quickly cooled on ice. In the next step, 12 μl of a solution containing 6 μl of 5× reverse transcriptase buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$ [GIBCO BRL]), 0.5 μl of 200 RNase inhibitor (200 U/ml; GIBCO BRL), 3 μl of 100 mM dithiothreitol, and 2.5 μl of 10 mM deoxynucleoside triphosphate was added, and the tubes were incubated for 2 min at 37°C. We then added 1.0 μl of Moloney murine leukemia virus reverse transcriptase (200,000 U/ml; GIBCO BRL) and incubated the sample for 60 min at 37°C. After receiving 45 μl of 0.7 M NaOH and 40 mM EDTA, the tubes were incubated for 10 min at 65°C and quickly cooled on ice. The resultant cDNA was precipitated with 75% ethanol overnight at −70°C. The precipitates were washed once with 75% ethanol, dried, and resuspended in 50 μl of diethylpyrocarbonate-treated distilled water. The samples were stored at −20°C until use. This reaction was always performed simultaneously for parallel samples from one experiment.

PCR was carried out in an automatic DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). For amplification of the desired cDNA, gene-specific primers (Table 1) were used. We added 1.0 μl of the sample cDNA solution to 49 μl of the reaction mixture, which contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 μg of gelatin per ml, deoxynucleoside triphosphates (each at a concentration of 200 μM), 1.0 μM sense and antisense primer, and 1.25 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). The preparations in the microtubes were amplified by using a three-temperature PCR system usually consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. PCR conditions of denaturation at 94°C for 1 min, primer annealing at 60°C for 2 min, and extension at 72°C for 3 min were used for transforming growth factor β (TGF-β). The number of cycles was determined for samples not reaching the amplification plateau (32 cycles for hypoxanthine phosphoribosyltransferase [HPRT] and TGF-β; and 35 cycles for the others), as shown in Table 1. The PCR products were electrophoresed on 2% agarose gels, stained with 0.5 μg of ethidium bromide per ml, and observed with a UV transilluminator.

Measurement of cytokines in lung homogenates. Mice were sacrificed at days 7 and 14 of infection, and the lungs were excised and then homogenized in 2.0 ml of normal saline with Polytron (Kinematica AG, Littau, Switzerland) at 12,000 rpm for 60 s on ice. The homogenates were centrifuged at 1,600 × *g* for 10 min, and the supernatants were passed through 0.22-μm-pore-size Millipore filters and assayed for concentrations of IFN-γ, tumor necrosis factor alpha (TNF-α), IL-4, and IL-10, using enzyme-linked immunosorbent assay (ELISA) kits purchased from Genzyme Co. (Cambridge, Mass.) for IFN-γ and from Endogen, Inc. (Cambridge, Mass.), for the others. The detection limits of the assay were 125 pg/ml for IFN-γ, 50 pg/ml for TNF-α, 5 pg/ml for IL-4, and 1.5 U/ml for IL-10.

RESULTS

Expression of cytokine and iNOS mRNA in the lungs of mice infected with *C. neoformans*. In our recent study (23), we established a murine model of pulmonary and disseminated infection with a highly virulent strain of *C. neoformans*. In this model, the fungal cells were directly instilled into the lungs, representing the initial infection site. The microorganisms multiplied rapidly in the lungs, disseminated to the brains within 3 weeks, and induced 100% mortality within 3 to 6 weeks of infection. To examine the kinetics for production of cytokines and iNOS in the lungs after infection with *C. neoformans*, we examined the mRNA expression for Th1 cytokines (including IL-2, LT, and IFN-γ), Th2 cytokines (including IL-4, IL-6, and IL-10), macrophage-derived cytokines (including TNF-α, IL-1β, TGF-β, IL-12p40, and IGIF), and iNOS, which catalyzes the generation of nitric oxide (NO) from L-arginine (15) and mediates the cryptococcocidal activity of IFN-γ-activated macrophages (1, 45). Examination of the lungs was performed on days 1, 3, 7, and 14 after intratracheal instillation of the fungus. As shown in Fig. 1A, there was a marginal expression of IFN-γ mRNA only on day 7 or no expression of IL-2 and LT mRNAs at any time interval, while IL-6 and IL-10 mRNAs were detected on days 1 to 14 and IL-4 mRNA was detected on days 1, 7, and 14 after infection. Among the macrophage-derived cytokines, there was little or no expression of TNF-α, IL-12p40, and IGIF mRNAs, similar to Th1 cytokines, while the TGF-β mRNA was detected on day 1 and then diminished with time (Fig. 1B). The IL-1β mRNA was expressed in the lungs of infected phosphate-buffered saline (PBS)-treated mice on days 1, 3, and 7 (Fig. 1B). On the other hand, there was little or no iNOS mRNA expression in the lungs (Fig. 1C). In mice instilled intratracheally with normal saline (uninfected mice), mRNAs of all cytokines noted above and iNOS were not generated at any time interval, while the HPRT mRNA was detected at a constant level (data not shown).

Modification of cytokine and iNOS mRNA expression by IL-12. Recently, we demonstrated that administration of IL-12 promoted the clearance of *C. neoformans* from the lungs and prevented dissemination of the fungal organism into the brain, resulting in reduced mortality (23). To elucidate the mechanism of the effect of IL-12, we investigated whether IL-12 treatment influences the synthesis of cytokines and iNOS in infected lungs. As shown in Fig. 2A, treatment caused an increase in the expression of IL-2 mRNA on days 7 and 14 and of LT and IFN-γ mRNAs through the course of infection. In contrast, IL-12 had little effect on the production of IL-4 and IL-10 during the early period of infection (days 1 and 3). Surprisingly, IL-12 treatment enhanced the production of these cytokines during the late stage of infection (days 7 and 14). The expression of IL-6 mRNA was shifted to the later time in the IL-12-treated mice compared to PBS-treated mice. IL-12 treatment has been reported recently to suppress the development of a primary Th2 response to a protein-hapten conjugate (27) and IL-4 production in mice during infection caused by *Leishmania major* (49). Among the macrophage-derived cytokines, IL-12 treatment caused an increase in the expression of TNF-α from day 3 and of IL-12p40 and IGIF mRNAs through the course of infection (Fig. 2B). Furthermore, it also increased TGF-β mRNA expression in the late period of infection (days 7 and 14), similar to that observed with Th2 cytokines. The expression of IL-1β mRNA was enhanced through the course of infection compared with infected PBS-treated mice (Fig. 2B). In addition, iNOS mRNA was considerably induced by this treatment on days 3, 7, and 14 postinfection (Fig. 2C).

Detection of cytokines in lung homogenates by ELISA. To confirm the results obtained by RT-PCR, we measured the concentrations of IFN-γ, TNF-α, IL-4, and IL-10 by ELISA using lung homogenates obtained from infected PBS- and IL-12-treated mice. No IFN-γ and small amounts of TNF-α were detected in lung homogenates on days 7 and 14 postinfection. In contrast, IL-12 treatment caused an increase in the production of these cytokines (Table 2). On the other hand, small but significant amounts of IL-4 and IL-10 were detected during natural infection, and the production of these Th2 cytokines was significantly augmented by IL-12 treatment (Table 2). These cytokines were not detected in the lung homogenates obtained from uninfected animals (data not shown).

DISCUSSION

In this study, we examined the production of Th1 and Th2 cytokines in the lungs of mice throughout the course of pul-

TABLE 1. Sequences of the oligonucleotide primers used for PCR amplification of cytokine and iNOS mRNA, product size predicted, and number of cycles

mRNA	Primer sequence[a]	Product (bp)	No. of cycles
HPRT			
Sense	5′-GTTGGATACAGGCCAAGACTTTGTTG-3′	165	32
Antisense	5′-GATTCAACTTGCGCTCATCTTAGGC-3′		
IL-1β			
Sense	5′-GCAACTGTTCCTGAACTCA-3′	382	35
Antisense	5′-CTCGGAGCCTGTAGTGCAG-3′		
IL-2			
Sense	5′-AACAGCGCACCCACTTCAA-3′	442	35
Antisense	5′-TTGAGATGATGCTTTGACA-3′		
IL-4			
Sense	5′-TAGTTGTCATCCTGCTCTT-3′	404	35
Antisense	5′-CTACGAGTAATCCATTTGC-3′		
IL-6			
Sense	5′-TTCCTCTCTGCAAGAGACT-3′	532	35
Antisense	5′-TGTATCTCTCTGAAGGACT-3′		
IL-10			
Sense	5′-TCCTTAATGCAGGACTTTAAGGGTTACTTG-3′	256	35
Antisense	5′-GACACCTTGGTCTTGGAGCTTATTAAAATC-3′		
IL-12p40			
Sense	5′-CAGAAGCTAACCATCTCCTGGTTTG-3′	394	35
Antisense	5′-TCCGGAGTAATTTGGTGCTTCACAC-3′		
IFN-γ			
Sense	5′-AACGCTACACACTGCATCT-3′	342	35
Antisense	5′-TGCTCATTGTAATGCTTGG-3′		
TNF-α			
Sense	5′-GGCAGGTCTACTTTGGAGTCATTGC-3′	308	35
Antisense	5′-ACATTCGAGGCTCCAGTGAATTCGG-3′		
LT			
Sense	5′-TCAGAAGCACTTGACCCAT-3′	322	35
Antisense	5′-AAGTCCCGGATACACAGACT-3′		
TGF-β			
Sense	5′-TGGACCGCAACAACGCCATCTATGAGAAAACC-3′	525	32
Antisense	5′-TGGAGCTGAAGCAATAGTTGGTATCCAGGGCT-3′		
IGIF			
Sense	5′-ACTGTACAACCGCAGTAATACGG-3′	434	35
Antisense	5′-AGTGAACATTACAGATTTATCCC-3′		
iNOS			
Sense	5′-CATGGCTTGCCCCTGGAAGTTTCTCTTCAAAG-3′	754	35
Antisense	5′-GCAGCATCCCCTCTGATGGTGCCATCG-3′		

[a] Primer sequences were obtained from previous reports by Montgomery and Dallman (30) for IL-1β, IL-2, IL-4, IL-6, IFN-γ, and LT, by Romani et al. (35) for IL-10, by Yoshida et al. (51) for IL-12p40, by Murray et al. (32) for TNF-α, by Chang et al. (4) for TGF-β, by Okamura et al. (34) for IGIF, and by Gazzinelli et al. (11) for HPRT and iNOS.

monary and disseminated infection with *C. neoformans* and found little or no production of Th1 cytokines (IL-2, LT, and IFN-γ) but considerable formation of Th2 cytokines (IL-4, IL-6, and IL-10). Furthermore, there was little or no production of macrophage-derived cytokines (TNF-α, IL-12p40, and IGIF), while IL-1β and TGF-β mRNAs were detected in considerable amounts during infection. Our results also demonstrated a lack of iNOS mRNA synthesis in infected lungs, consistent with the impaired generation of IFN-γ and TNF-α. On the other hand, the pulmonary production of Th1 cytokines, TNF-α, IL-12p40, IGIF, and iNOS was augmented following administration of IL-12. Interestingly, similar results were obtained regarding the effect of IL-12 treatment on synthesis of IL-4, IL-10, and TGF-β.

Several studies emphasized the importance of Th1 cytokines in host defense mechanisms against infection caused by various microbial pathogens (reviewed by Scott and Kaufmann [40]). In particular, IFN-γ is considered to play a critical role in the activation of macrophages through induction of NO, a principal mediator for macrophage killing activity in mice (33). In

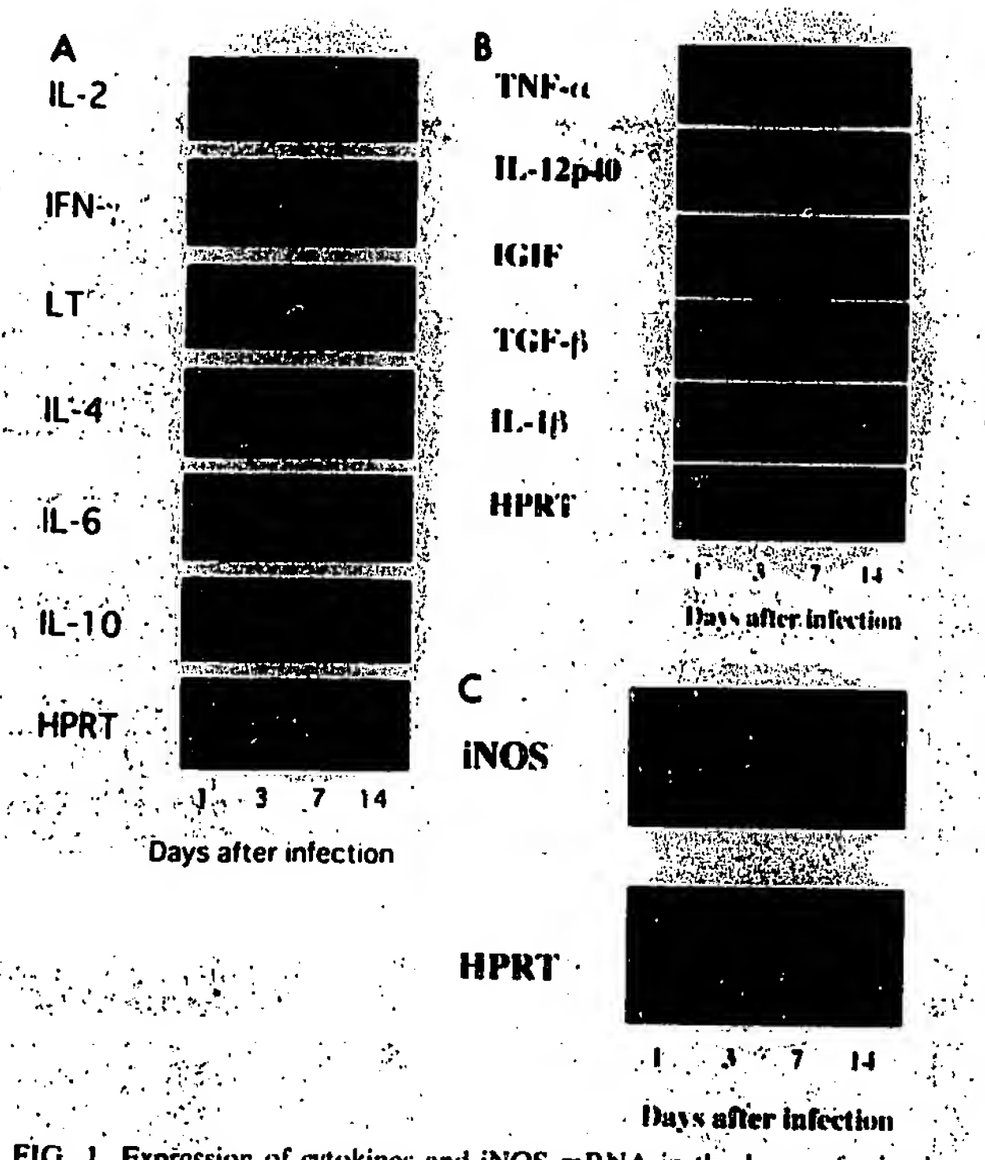


FIG. 1. Expression of cytokines and iNOS mRNA in the lungs of mice infected with *C. neoformans*. Mice received daily intraperitoneal injections of 200 μl of PBS for 7 days from the day of intratracheal instillation of 10^5 cells of *C. neoformans*. On days 1, 3, 7, and 14 of instillation, mice were sacrificed and total RNA was extracted from their lungs. Subsequently, RT-PCR was carried out for Th1 and Th2 cytokines (A), macrophage-derived cytokines (B), and iNOS (C). HPRT was used as an internal control. The PCR products were electrophoresed on 2% agarose gels, stained with 0.5 μg of ethidium bromide per ml, and observed with a UV transilluminator. The RNA samples were obtained from three mice in each group, and the results are representative of three separate samples.

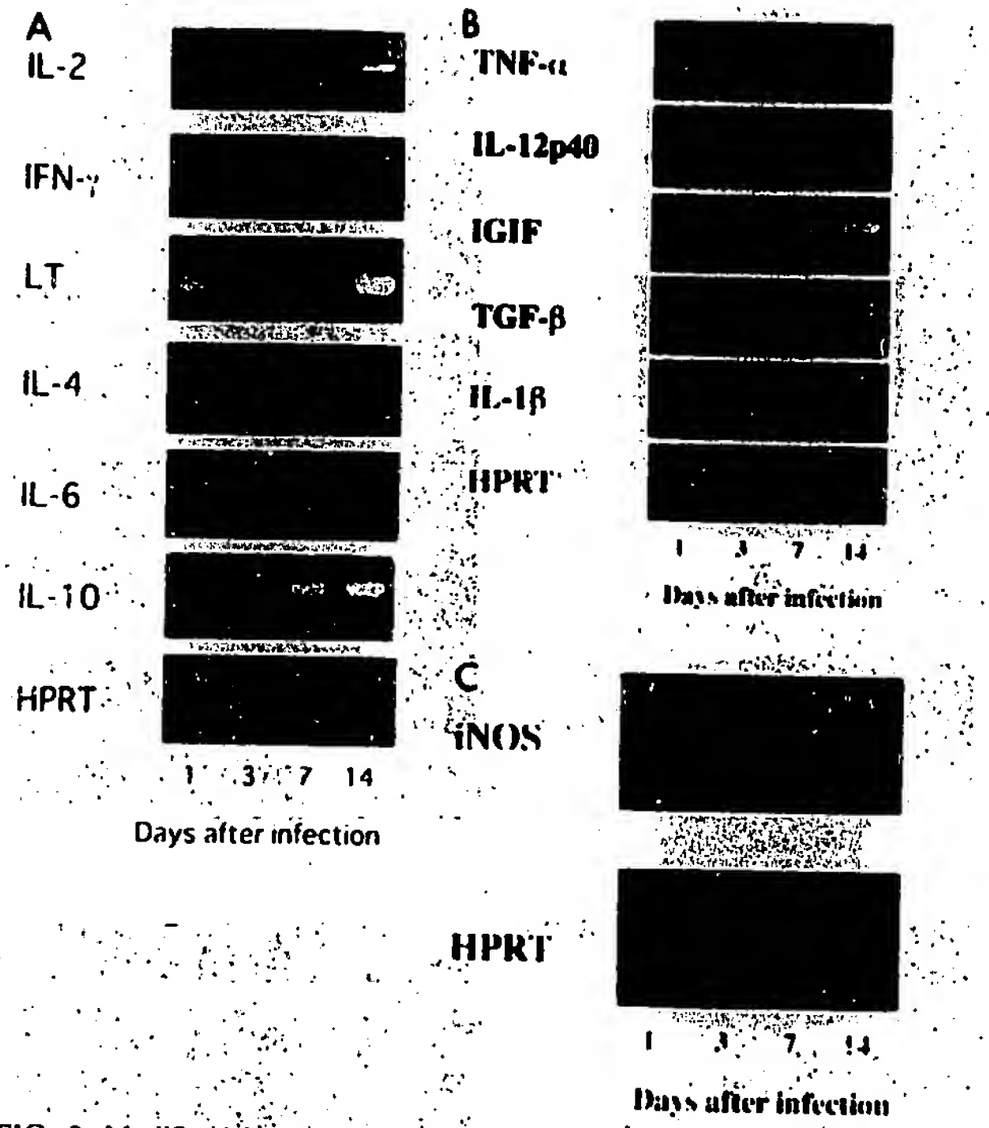


FIG. 2. Modification of cytokines and iNOS mRNA expression by IL-12. Mice received daily intraperitoneal injections of recombinant murine IL-12 (0.1 μg/mouse) for 7 days from the day of intratracheal instillation of 10^5 cells of *C. neoformans*. On days 1, 3, 7, and 14 of instillation, mice were sacrificed and total RNA was extracted from their lungs. Subsequently, RT-PCR was carried out for Th1 and Th2 cytokines (A), macrophage-derived cytokines (B), and iNOS (C). HPRT was used as an internal control. The PCR products were electrophoresed on 2% agarose gels, stained with 0.5 μg of ethidium bromide per ml, and observed with a UV transilluminator. The RNA samples were obtained from three mice in each group, and the results are representative of three separate samples.

addition, IFN-γ enhances the antigen-presenting activity of macrophages, resulting in the expansion of Th1 cell population (21). The activated Th1 cells in turn produce large amounts of IFN-γ and effectively augment macrophage antimicrobial activity. These biological responses may establish a positive feedback circuit to promote host resistance to microbial infection. In our model of pulmonary and disseminated infection with *C. neoformans*, all mice died within 4 to 6 weeks due to the rapid multiplication of cryptococci in the lungs and its dissemination to the brain (23). Therefore, the present results indicate that insufficient production of Th1 cytokines and iNOS in the infected lungs may account for the uncontrolled infection with *C. neoformans*.

In contrast, induction of TGF-β and Th2 cytokines, including IL-4 and IL-10, became evident during the early stages of infection. Previous studies showed that constitutive production of IL-4 in transgenic mice resulted in increased susceptibility to infection with *L. major* (25) and that resistance to infection occurs when the endogenously synthesized IL-4 is neutralized in the susceptible mice (5). IL-10 is reported to inhibit Th1 cell-mediated responses mostly by downregulating expression of major histocompatibility complex class II and CD80 on antigen-presenting macrophages (8, 9). The resistance of mice to infection with various microorganisms is also impaired by administration of recombinant IL-10 (10, 42). Furthermore, administration of a neutralizing antibody enhances the resistance to microbial infection (7). Similarly, treatment with TGF-β results in failure of animals to protect themselves against infection caused by *Toxoplasma gondii*, probably due to suppression of IFN-γ production (19). These observations indicate that production of greater amounts of Th2 than of Th1 cytokines and the generation of TGF-β, as shown in the present study, may be closely correlated with the lethal pulmonary and disseminated infection with *C. neoformans* in our

TABLE 2. Cytokine levels in lung homogenates

Cytokine	Level[a]			
	7 days		14 days	
	PBS/infected[b]	IL-12/infected[c]	PBS/infected	IL-12/infected
IFN-γ	ND[d]	526 ± 73.1	ND	861.3 ± 347.0
TNF-α	91.5 ± 30.8	1,623 ± 574.6	74.3 ± 19.2	1,306.4 ± 225.8
IL-4	8.2 ± 1.7	13.8 ± 7.5	6.1 ± 0.5	15.2 ± 4.1
IL-10	4.9 ± 1.1	16.2 ± 3.8	5.3 ± 2.4	31.5 ± 8.1

[a] The concentration of each cytokine in lung homogenate was measured by ELISA. Levels of IFN-γ, TNF-α, and IL-4 are in picograms/milliliter; levels of IL-10 are in units/milliliter. Each value is the mean ± standard deviation for three mice.

[b] Mice were infected intratracheally with *C. neoformans* but not treated with IL-12.

[c] Mice infected with *C. neoformans* received daily intraperitoneal injections of recombinant IL-12 (0.1 μg/mouse/day) for 7 days.

[d] ND, not detected.

model. To confirm this possibility, further studies with neutralizing antibodies against these cytokines will be necessary.

IL-12 enhances host defense against infection caused by microbial pathogens (12, 14, 23, 47). Studies with cultured cells have demonstrated that IL-12 induces the production of IFN-γ by NK cells, γδT cells, and conventional T cells (43, 46) and potentiates the development of Th1 cells from naive cells (46). In addition, it suppresses the generation of Th2 cytokines and their biological activities through the induction of IFN-γ (46). These observations suggest that IL-12 treatment may suppress the in vivo generation of Th2 cytokines in our experimental murine model, thus protecting these animals from death. However, the results showed that the expression of Th2 cytokine mRNA was markedly enhanced, particularly in the late stage of infection. In addition, the same treatment enhanced the expression of TGF-β mRNA, a potent immunosuppressive cytokine (36, 39, 41). The mechanism by which IL-12 augments the generation of Th2 cytokines and TGF-β in the lungs remains to be elucidated. Recently, several investigators (20, 28, 38, 50) have reported similar observations, showing that IL-12 enhanced the production of IL-4 and IL-10 by Th0 and Th2 cells.

We demonstrated in our recent study (23) that IL-12 treatment induced marked cellular inflammatory changes in the lungs of mice during the early stage of pulmonary and disseminated cryptococcosis. Our unpublished results further showed that no live *C. neoformans* was detected and the inflammatory changes subsided in the lungs 9 weeks after infection in those mice that survived the infection. These observations suggest that some mechanism operates to attenuate persistent inflammatory reactions, which may be harmful to host. In previous studies (13, 24), systemic injection of endotoxin was demonstrated to induce the fatal hyperinflammatory responses through overproduction of cytokines such as TNF-α and IFN-γ. In this endotoxemia model, administration of IL-10 protected animals from the lethal endotoxin shock, probably through suppressing the production of TNF-α and IFN-γ (26). Similarly to their findings, in our model of cryptococcal infection, persistent activation of Th1 cell-mediated responses induced by IL-12 could result in the fatal outcome due to hyperinflammatory responses. However, no mice died because of this mechanism. Considered together, these findings indicate there may be a certain negative feedback mechanism operating through Th2 cytokines and TGF-β to prevent uncontrolled Th1 responses, as suggested by Meyaard et al. (28). Further studies are necessary to examine this possibility.

IGIF induces the production of IFN-γ, proliferation of T cells, and activation of NK cells, effects that are similar to those of IL-12 (34). Interestingly, the effects of IGIF on the induction of IFN-γ production paralleled those of IL-12 (34). At present, the biological significance of this newly identified cytokine remains unclear. Combining the data in the present study with those from our previous study (23), we conclude that IGIF synthesis appears to be correlated with protection of mice from cryptococcal infection. This may suggest that IGIF plays a role in host defense to pathogenic organisms. Furthermore, the induction of both IL-12 and IGIF synthesis following treatment with IL-12 may support a possible positive feedback mechanism involved in the generation of IFN-γ and resulting in the potentiation of the protective effects.

REFERENCES

1. **Alspaugh, J. A., and D. L. Granger.** 1991. Inhibition of *Cryptococcus neoformans* replication by nitrogen oxides supports the role of these molecules as effectors of macrophage-mediated cytostasis. Infect. Immun. 59:2291–2296.
2. **Belosevic, M. D., S. Finbloom, P. H. Van Der Meide, M. V. Slayter, and C. A. Nacy.** 1989. Administration of monoclonal anti-IFN-gamma antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major*. J. Immunol. 143:266–274.
3. **Brummer, E., and D. Stevens.** 1994. Anticryptococcal activity of macrophages: role of mouse strain, C5, contact, and L-arginine. Cell. Immunol. 157:1–10.
4. **Chang, C. M., A. Limanni, W. H. Baker, M. E. Dobson, J. F. Kalinich, W. Jackson, and M. L. Patchen.** 1995. Bone marrow and splenic granulocyte-macrophage colony-stimulating factor and transforming growth factor-β mRNA levels in irradiated mice. Blood 86:2130–2136.
5. **Chatelain, R., K. Varkila, and R. L. Coffman.** 1992. IL-4 induces a Th2 response in Leishmania major-infected mice. J. Immunol. 148:1182–1187.
6. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159.
7. **Denis, M., and E. Champsi.** 1993. IL-10 neutralization augments mouse resistance to systemic *Mycobacterium avium* infections. J. Immunol. 151: 5425–5432.
8. **de Waal Malefyt, R., J. Haanen, H. Spits, M.-G. Roncarolo, A. te Velde, C. Figdor, K. Jhonson, R. Kastelein, H. Yssel, and J. E. de Vries.** 1991. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J. Exp. Med. 174:915–924.
9. **Ding, L., P. S. Linsley, L.-Y. Huang, R. N. Germain, and E. M. Shevach.** 1993. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. J. Immunol. 151:1224–1234.
10. **Fernandes, D. M., and C. L. Baldwin.** 1995. Interleukin-10 downregulates protective immunity to *Brucella abortus*. Infect. Immun. 63:1130–1133.
11. **Gazzinelli, R., I. Eltoum, T. A. Wynn, and A. Sher.** 1993. Acute cerebral toxoplasmosis is induced by in vivo neutralization of TNF-α and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. J. Immunol. 151:3672–3681.
12. **Gazzinelli, R. T., M. Wysocka, S. Hayashi, E. Y. Denkers, S. Hieny, P. Caspar, G. Trinichieri, and A. Sher.** 1994. Parasite-induced IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with *Toxoplasma gondii*. J. Immunol. 153:2533–2543.
13. **Heinzel, F. P.** 1990. The role of IFN-gamma in the pathology of experimental endotoxemia. J. Immunol. 145:2920–2924.
14. **Heinzel, F. P., D. S. Schoenhaut, R. M. Rerko, L. E. Rosser, and M. K. Gately.** 1993. Recombinant interleukin 12 cures mice infected with *Leishmania major*. J. Exp. Med. 177:1505–1509.
15. **Hibbs, J. B., Jr., R. R. Taintor, and Z. Vavrin.** 1987. Macrophage cytotoxicity: role for L-arginine deaminase and amino nitrogen oxidation to nitrite. Science 197:473–476.
16. **Hill, J. O., and A. G. Harmsen.** 1991. Intrapulmonary growth and dissemination of an avirulent strain of *Cryptococcus neoformans* in mice depleted of CD4+ or CD8+ T cells. J. Exp. Med. 173:755–758.
17. **Hsieh, C. S., S. E. Macatonia, C. S. Tripp, A. O'Garra, and K. M. Murphy.** 1993. Development of Th1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 260:547–549.
18. **Huffnagle, G. B., J. L. Yates, and M. F. Lipscomb.** 1991. Immunity to a pulmonary *Cryptococcus neoformans* infection requires both CD4+ and CD8+ T cells. J. Exp. Med. 173:793–800.
19. **Hunter, C. A., L. Bermudez, H. Beernink, W. Waegell, and J. S. Remington.** 1995. Transforming growth factor-β inhibits interleukin-12-induced production of interferon-γ by natural killer cells: a role for transforming growth factor-β in the regulation of T cell-independent resistance to *Toxoplasma gondii*. Eur. J. Immunol. 25:994–1000.
20. **Jeannin, P., Y. Delneste, P. Life, J.-F. Gauchat, D. Kaiserlian, and J.-Y. Bonnefoy.** 1995. Interleukin-12 increases interleukin-4 production by established human Th0 and Th2-like T cell clones. Eur. J. Immunol. 25:2247–2252.
21. **Kawakami, K., Y. Yamamoto, K. Kakimoto, and K. Onoue.** 1989. Requirement for delivery of signals by physical interaction and soluble factors from accessory cells in the induction of receptor-mediated T cell proliferation. Effectiveness of IFN-γ modulation of accessory cells for physical interaction with T cells. J. Immunol. 142:1818–1825.
22. **Kawakami, K., M. Tohyama, K. Teruya, N. Kudeken, Q. Xie, and A. Saito.** 1996. Contribution of interferon-γ in protecting mice during pulmonary and disseminated infection with *Cryptococcus neoformans*. FEMS Immunol. Med. Microbiol. 13:123–130.
23. **Kawakami, K., M. Tohyama, Q. Xie, and A. Saito.** 1996. Interleukin-12 protects mice against pulmonary and disseminated infection caused by *Cryptococcus neoformans*. Clin. Exp. Immunol. 104:208–214.
24. **Kiener, P. A., F. Marek, G. Rodgers, P. F. Lin, G. Warr, and J. Desiderio.** 1988. Induction of tumor necrosis factor, IFN-gamma, and acute lethality in mice by toxic and non-toxic forms of lipid A. J. Immunol. 141:870–874.
25. **Leal, L., D. Moss, R. Kuhn, W. Muller, and F. Liew.** 1993. Interleukin-4 transgenic mice of resistant background are susceptible to *Leishmania major* infection. Eur. J. Immunol. 23:566–569.
26. **Marchant, A., C. Bruyns, P. Vandenabeele, M. Ducarme, C. Gerard, A. Delvaux, D. De Groote, D. Abramowicz, T. Velu, and M. Goldman.** 1994.

Interleukin-10 controls interferon-gamma and tumor necrosis factor production during experimental endotoxemia. Eur. J. Immunol. 24:1167–1171.
27. **Mcknight, A. J., G. J. Zimmer, I. Fogelman, S. F. Wolf, and A. K. Abbas.** 1994. Effects of IL-12 on helper T cell-dependent immune responses in vivo. J. Immunol. 152:2172–2179.
28. **Meyaard, L., E. Hovenkamp, S. A. Otto, and F. Miedema.** 1996. IL-12-induced IL-10 production by human T cells as a negative feedback for IL-12-induced immune responses. J. Immunol. 156:2776–2782.
29. **Mody, C. H., M. F. Lipscomb, N. E. Street, and G. B. Toews.** 1990. Depletion of CD4+ (L3T4+) lymphocytes in vivo impairs murine host defense to *Cryptococcus neoformans*. J. Immunol. 144:1472–1477.
30. **Montgomery, R., and M. J. Dallman.** 1991. Analysis of cytokine gene expression during fetal thymic ontogeny using the polymerase chain reaction. J. Immunol. 147:554–560.
31. **Mosmann, T. R., and S. Sad.** 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol. Today 17:138–146.
32. **Murray, L. J., R. Lee, and C. Martens.** 1990. In vivo cytokine gene expression in T cell subsets of the autoimmune MRL/Mp-*lpr/lpr* mouse. Eur. J. Immunol. 20:163–170.
33. **Nathan, C.** 1992. Nitric oxide as a secretory product of mammalian cells. FASEB J. 6:3051–3064.
34. **Okamura, H., H. Tsutsui, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, K. Akita, M. Namba, F. Tanabe, K. Konishi, S. Fukuda, and M. Kurimoto.** 1995. Cloning of a new cytokine that induces IFN-γ production by T cells. Nature 378:88–91.
35. **Romani, L., A. Mencacci, L. Tonnetti, R. Spaccapelo, E. Cenci, P. Puccetti, S. F. Wolf, and F. Bistoni.** 1994. IL-12 is both required and prognostic in vivo for T helper type 1 differentiation in murine candidiasis. J. Immunol. 153: 5167–5175.
36. **Ruegemer, J. J., S. N. Ho, J. A. Augustine, J. W. Schlager, M. P. Bell, D. J. McKean, and R. T. Abraham.** 1990. Regulatory effects of transforming growth factor β on IL-2- and IL-4-dependent T cell cycle progression. J. Immunol. 144:1767–1776.
37. **Salkowski, C. A., and E. Balish.** 1991. A monoclonal antibody to gamma interferon blocks augmentation of natural killer cell activity induced during systemic cryptococcosis. Infect. Immun. 59:486–493.
38. **Schmitt, E., P. Hoehn, T. Germann, and E. Rude.** 1994. Differential effects of interleukin-12 on the development of naive mouse CD4+ T cells. Eur. J. Immunol. 24:343–347.
39. **Schmitt, E., P. Hoehn, C. Huels, S. Goedert, N. Palm, E. Rude, and T. Germann.** 1994. T helper type 1 development of naive CD4+ T cells requires the cordinate action of interleukin-12 and interferon-γ and is inhibited by transforming growth factor-β. Eur. J. Immunol. 24:793–798.
40. **Scott, P., and S. H. E. Kaufmann.** 1991. The role of T-cell subsets and cytokines in the regulation of infections. Immunol. Today 12:346–348.
41. **Siepl, C., S. Bodmer, K. Frei, H. R. MacDonald, R. DeMartin, E. Hofer, and A. Fontana.** 1988. The glioblastoma-derived T cell suppression factor/transforming growth factor-β2 inhibits T cell growth without affecting the interaction of interleukin 2 with its receptor. Eur. J. Immunol. 18:593–600.
42. **Silva, J. S., P. J. Morrissey, K. H. Grabstein, K. M. Mohler, D. Anderson, and S. G. Reed.** 1992. Interleukin 10 and interferon γ regulation of experimental *Trypanozoma cruzi* infection. J. Exp. Med. 175:169–174.
43. **Skeen, M. J., and H. K. Ziegler.** 1995. Activation of γδ T cells for production of IFN-γ is mediated by bacteria via macrophage-derived cytokines IL-1 and IL-12. J. Immunol. 154:5832–5841.
44. **Stevens, D. A.** 1990. Fungal infections in AIDS patients. Br. J. Clin. Practice 44(Suppl.1):11–22.
45. **Tohyama, M., K. Kawakami, M. Futenma, and A. Saito.** 1996. Enhancing effect of oxygen radical scavengers on anticryptococcal activity of murine peritoneal macrophages through production of nitric oxide. Clin. Exp. Immunol. 103:436–441.
46. **Trinchieri, G.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. 13:251–276.
47. **Tripp, C. S., M. K. Gately, J. Hakimi, P. Ling, and E. R. Unanue.** 1994. Neutralization of IL-12 decreases resistance to *Listeria* in SCID and CB-17 mice. J. Immunol. 152:1883–1887.
48. **Ushio, S., M. Namba, T. Okura, K. Hattori, Y. Nukada, K. Akita, F. Tanabe, K. Konishi, M. Micallef, M. Fujii, K. Torigoe, T. Tanimoto, S. Fukuda, M. Ikeda, H. Okamura, and M. Kurimoto.** 1996. Cloning of the cDNA for human IFN-γ-inducing factor, expression in *Escherichia coli*, and studies on the biologic activities of the protein. J. Immunol. 156:4274–4279.
49. **Wang, Z., S. Zheng, D. B. Corry, D. K. Dalton, R. A. Seder, S. L. Reiner, and R. M. Locksley.** 1994. Interferon γ-independent effects of interleukin 12 administered during acute or established infection due to *Leishmania major*. Proc. Natl. Acad. Sci. USA 91:12932–12936.
50. **Wynn, T. A., D. Jankovic, S. Hieny, K. Zioncheck, P. Jardieu, A. W. Cheever, and A. Sher.** 1995. IL-12 exacerbates rather than suppresses T helper 2-dependent pathology in the absence of endogenous IFN-γ. J. Immunol. 154: 3999–4009.
51. **Yoshida, A., Y. Koide, M. Uchijima, and T. O. Yoshida.** 1994. IFN-γ induces IL-12 mRNA expression by a murine macrophage cell line, J774. Biochem. Biophy. Res. Commun. 198:857–861.

Editor: T. R. Kozel

QR180.J6
+
MIC

STIC-ILL

From: Prasad, Sarada
Sent: Tuesday, March 06, 2001 5:33 PM
To: STIC-ILL

Please fetch me the following full text citations for the case # 09,395,038

1. Philosophical transactions of the roayal Society of London B Biological Sciences (Sept. 29, 1997) vol. 352, No. 1359, pages 1311-1315.(cytokines and nitric oxide as effector molecules against parasitic infections.
2. J Immunol. 1994, vol.153, No.11, pages 5200-5209. (Elevated expression of Th1 cytokines and nitric oxide synthase in the lungs of vaccinated mice after challenge infection with schistosoma mansoni.)
3. Cancer immunol, Immunotherpy, 1996 jan, vol.42, No.1, pages 38-46. (Effects of N-methyl arg, an inhibitor of nitric oxide synthesis on IL-2 induced capillary leakage and anti-tumor responses innhealthy and tumor bearing mice.
4. Infection and immunity 1997, vol. 65, No. 4 1307-1312. (Expression of cytokines and inducible nitric oxuide synthase mRNA in the lungs of mice infected with cryptococcus neoformans: effects of interleukin 12).
5. Journal of general virology 1998, vol.79, No. 4, pages 825-830 (Mice lacking inducible niric oxide synthase are more susceptible to herpers simplex virus infection despite enhanced Th1 cells responses)
6. J Neuroimmunology 1996, vol.68, No. 1-2, pages 101-108. (Interferon gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons.)
7. J Experimental medicine 1996, vol.183, No. 4, pages 1447-1459. (The role of interleukin 12 and nitric oxide in the development of spontaneous auroimmune disese in mrl...mice).

thank you,

Sarada Prasad, Ph.D.
Patent Examiner
Art Unit 1646
Room CM1-8B17
Tel: 703-305-1009

Elevated Expression of Th1 Cytokines and Nitric Oxide Synthase in the Lungs of Vaccinated Mice After Challenge Infection with *Schistosoma mansoni*

Thomas A. Wynn,[1]* Isabelle P. Oswald,* Isam A. Eltoum,* Patricia Caspar,* Charles J. Lowenstein,† Fred A. Lewis,‡ Stephanie L. James,* and Alan Sher*

*Immunology and Cell Biology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892; †Department of Neuroscience, Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205; and ‡The Biomedical Research Institute, Rockville, MD 20852

C57BL/6 mice were vaccinated with irradiated cercariae of *Schistosoma mansoni*, and, at various times after challenge infection, total lung mRNA was isolated to assess the induction of several cytokines that previously had been shown in in vitro studies to be involved in the activation of macrophages and/or endothelial cells for nitric oxide (NO) production and killing of schistosomula. Vaccinated mice demonstrated a highly significant increase in IFN-γ mRNA upon subsequent infection when compared with infected nonvaccinated controls. A similar, although less dramatic, increase in two other macrophage-activating cytokines, TNF-α and IL-2, also was observed. In contrast, although the Th2 cytokines IL-4, IL-5, IL-10, and IL-13 were elevated in challenged vaccinated animals, only IL-10 and IL-13 showed increases that were significant with respect to the mRNA levels observed in challenged controls. Neutralization of IFN-γ reduced immunity in vaccinated animals and resulted in decreased IFN-γ, IL-2, IL-10, TNF-α, and IL-12 p40 but markedly increased IL-4, IL-5, and IL-13 mRNA expression and serum IgE levels. Pulmonary NO synthase expression was elevated in immunized mice at a time at which immune elimination of schistosomula is believed to occur. Moreover, suppression of NO synthase activity with the inhibitor aminoguanidine reduced immunity, as measured by a 32 to 33% increase in worm burden. Together, these data support previous in vitro studies that suggest a role for NO in schistosomulum killing. Furthermore, the observation that the down-regulatory cytokines IL-4, IL-10, and IL-13 are induced together with IFN-γ may provide an explanation for the failure of this vaccine to provide complete protection. *The Journal of Immunology*, 1994, 153: 5200.

Schistosomiasis is a major parasitic disease that affects between two hundred million and three hundred million people worldwide (1). The development of an effective defined Ag vaccine has been a primary goal of immunologists studying schistosome infection. Success in this area has been limited by a lack of understanding of immune responses that are effective against the parasite in vivo. Numerous studies have implicated both cellular and humoral effector mechanisms in parasite attrition (2, 3). More recent work has focused on defining the Th cell subsets and cytokines underlying these protective immune responses.

An important animal model used in the study of schistosome immunity is the induction of resistance in mice by vaccination with radiation-attenuated cercariae. After a single exposure to attenuated cercariae, animals are able to eliminate 60 to 80% of the worms that ordinarily develop from a challenge infection. This resistance is dependent on $CD4^+$ T lymphocyte function but independent of $CD8^+$ cells, complement, IgM production, or IgE-mediated immediate-type hypersensitivity (4–8). Moreover, protective immunity seems to be associated with the function of the Th1, rather than Th2, subset of $CD4^+$ cells. Thus, treatment of vaccinated mice with Abs to IFN-γ causes a dramatic reduction in immunity, whereas Abs against the Th2 cytokines IL-4 and IL-5 have no effect (9, 10).

Several lines of evidence suggest that cell-mediated immunity involving IFN-γ-activated effector cells is the major mechanism of protective immunity in this model (11).

Received for publication April 21, 1994. Accepted for publication August 23, 1994.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Thomas A. Wynn, 9000 Rockville Pike, National Institutes of Health, Building 4, Room 126, Bethesda, MD 20892.

0022-1767/94/$02.00

Indeed, P strain mice, which are defective in macrophage activation, fail to develop significant levels of immunity after immunization with irradiated cercariae (12). Moreover, schistosomula are highly susceptible to killing in vitro by cytokine-activated macrophages or endothelial cells (13). Although IFN-γ is a major activating cytokine in this system, TNF-α, IL-1, and IL-2 also stimulate larvicidal activity (11). The immune effector mechanism of parasite killing involves the arginine-dependent production of toxic, reactive nitrogen intermediates (NOs)[2] (14, 15), and is inhibited by NO synthase inhibitors or by the addition of excess iron to the culture medium (16).

In vivo studies indicate that the lungs are the major site of attrition of challenge parasites in mice vaccinated with irradiated cercariae (17), and that schistosomulum elimination is associated with increased pulmonary T lymphocyte response and mononuclear cell inflammation (18). In this study, to better understand the mechanisms that control parasite attrition in vivo, we have characterized the evolving pulmonary cytokine mRNA and protein patterns in animals vaccinated and challenged with *Schistosoma mansoni* cercariae, with special emphasis being placed on cytokines that previously have been shown to activate macrophages and endothelial cells for larval killing (e.g., IFN-γ, TNF-α, IL-1, and IL-2) or to suppress this activity (IL-4 and IL-10) (13, 15). In addition, we used in vivo depletion studies to examine both the effects of IFN-γ on cytokine patterns and NO synthase expression within the lung and the direct contribution of NO to parasite killing.

Materials and Methods

Laboratory hosts and parasites

Six-week-old female C57BL/6 mice were purchased from the Division of Cancer Treatment, National Cancer Institute, Frederick, MD. Cercariae of *S. mansoni* (NMRI strain) were obtained from infected *Biomphalaria glabrata* snails (Biomedical Research Institute, Rockville, MD). In some experiments, schistosomula were prepared from cercariae by mechanical transformation and gradient purification (19). Soluble worm Ag preparation (SWAP) was derived from homogenized adult parasites as described previously (20).

Immunizations and infections

S. mansoni cercariae were attenuated with 50,000 rad of γ-irradiation from a ^{137}Cs source. Mice were vaccinated by immersing the tail in water that contained 500 irradiated cercariae for 40 min. Vaccinated mice or age- and sex-matched controls were used 4 to 5 wk after vaccination, when they routinely display highly significant levels of immunity (21, 22). Mice were challenged percutaneously with 120 cercariae in all immunity studies and with 500 cercariae for cytokine mRNA experiments. When animals were perfused 6 wk later so that the degree of protective immunity could be determined, vaccinated mice routinely displayed a highly significant ($p < 0.001$) reduction in worm burdens as compared with controls. The level of resistance for vaccinated mice was calculated from the mean worm burdens of control mice by using the following formula: percent resistance R = (control − vaccinated)/control × 100. The statistical significance between worm burdens was evaluated by using Student's *t*-test.

[2] Abbreviations used in this paper: NO, nitric oxide; RT, reverse transcriptase; SWAP, soluble worm Ag preparation; AMG, aminoguanidine hemisulfate.

Ab and cytokine reagents

A neutralizing mAb that was specific for murine IFN-γ (XMG 1.6) and a rat IgG control (GL113) that was directed against *Escherichia coli* β-galactosidase were purified by precipitation from ascites with ammonium sulfate. Mice were treated with anti-IFN-γ or control mAbs (1 mg/mouse/i.p. injection) during the vaccination period; Ab was administered on days −1, 6, 12, 19, and 26 and on days −1, 3, 7, 11, and 15 postchallenge.

Intravenous challenge of schistosomula

The induction of synchronous reactions to injected schistosomula was performed as described previously (23). Briefly, cercariae were mechanically transformed and incubated for 24 h in RPMI plus 5% FCS at 37°C in 6% CO_2. Schistosomula were washed three times and resuspended in PBS before i.v. injection. Animals were killed 1 to 14 days after injection, and the right lung was placed in 1 ml of RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) and frozen on dry ice in preparation for RNA extraction. In some experiments, the left lung was prepared for immunohistochemical studies.

Lymphocyte culture

Cells from the lymph nodes that drain the lung (mediastinal and thoracic para-aortic) were cultured at 10^7 cells/ml for lymphokine production in RPMI 1640 (Biofluids, Rockville, MD), which contained 10% heat-inactivated FCS (Sterile Systems, Inc. Logan, UT), 2 mM glutamine, 100 U penicillin, 100 μg streptomycin/ml, 10 mM HEPES, and 5×10^5 M 2-ME. Cells were incubated with SWAP at 50 μg/ml or Con A at 5 μg/ml (20). Supernatant fluids were collected at 72 h for lymphokine assays.

Lymphokine and Ab assays

As described previously, supernatant fluids were assayed for IFN-γ by two-site ELISA using mAb HB170 and a polyclonal rabbit anti-mouse rIFN-γ (24, 25) standardized against mouse rIFN-γ control (gift of Genentech, South San Francisco, CA). IL-5 was assayed by ELISA using two mAbs that recognize distinct epitopes (TRFK5 and TRFK4) (26) and standardized against murine rIL-5 (all reagents were gifts of Dr. Robert Coffman, DNAX, Palo Alto, CA). In some experiments, mice were bled by orbital puncture on days 7 to 28 after challenge. Levels of total serum IgE Abs were quantified by ELISA as described previously (27).

Immunohistochemistry

Five-micrometer frozen sections in O.C.T. compound (Miles Inc., Elkhart, IN) were prepared from lungs that had been inflated and fixed with 4% buffered paraformaldehyde (Ted Pella, Inc., Redding, CA) immediately after animals were killed. Sections were preincubated with PBS containing 0.05% Tween 20 plus 1% BSA for 30 min and washed three times with 0.05% Tween after each incubation. Endogenous peroxidase was blocked with 3% H_2O_2 prepared in absolute methanol. After this nonspecific blocking step, slides were incubated overnight with a polyclonal rabbit anti-mouse macrophage-NO synthase (1:100 dilution). Adjacent sections were incubated with control antiserum. This incubation was followed by a 20-min incubation with a biotin-labeled donkey anti-rabbit Ab (The Jackson Laboratory, Bar Harbor, ME) at a 1:500 dilution. Peroxidase-conjugated streptavidin (BioGenex Laboratories, San Ramon, CA) was added and incubated for 30 min, then substrate Chromagen (BioGenex) was added.

Inhibition of NO synthase activity

For in vivo inhibition of NO synthase, aminoguanidine hemisulfate (AMG) salt (Sigma Chemical Co., St. Louis, MO) was used as described previously (28). Mice were injected twice daily with 500 mg AMG/kg

body weight, which had been diluted in 0.5 ml PBS for 4 wk after challenge with cercariae.

RT-PCR detection of cytokine mRNAs

RNase-free plastic and water were used throughout. The right lung was homogenized in 1 ml RNA STAT-60 by using a tissue polytron (Omni International, Waterbury, CT), and total RNA was isolated as recommended by the manufacturer. The RNA was resuspended in diethylpyrocarbonate-treated water that contained 1 mM EDTA and quantified spectrophotometrically. A RT-PCR procedure was performed, as described previously (29), to determine relative quantities of mRNA for IL-2, IL-4, IL-5, IL-10, IL-13, IFN-γ, IL-12 (p35 and p40 subunits), TNF-α, IL-1β, inducible nitric oxide synthase (30), and hypoxanthine phosphoribosyltransferase. The primers and probes for all genes have been published previously (29, 31–33). PCR conditions were strictly defined for each cytokine primer pair, such that a linear relationship between input RNA and final PCR product was obtained. Both positive and negative controls were included in each assay to confirm that only cDNA PCR products were detected, and that none of the reagents were contaminated with cDNA or previous PCR products. The number of PCR cycles selected for each gene were as follows: IL-2, 33 cycles; IL-4, 32 cycles; IL-5, 31 cycles; IL-10, 32 cycles; IL-13, 30 cycles; IFN-γ, 28 cycles; p35, 30 cycles; p40, 33 cycles; TNF-α, 33 cycles; IL-1β, 26 cycles; inducible nitric oxide synthase, 33 cycles; and hypoxanthine phosphoribosyltransferase, 21 cycles. After the appropriate number of PCR cycles, the amplified DNA was analyzed by electrophoresis, Southern blotting, and hybridization with cytokine-specific probes as described previously (29). The chemiluminescent signals were quantified by using a 600 ZS scanner (Microtek International, Torrance, CA). The amount of PCR product was determined by comparing a signal density with that of standard curves generated from simultaneously amplified step-wise dilutions of cDNA obtained from samples with high amounts (as determined from initial experiments) of specific cytokine mRNA. Fold increase was calculated as the reciprocal of the equivalent dilution of control (unchallenged mouse lung) cDNA. The IL-12 p35 chain was constitutively expressed in the lungs of all animals and was not significantly modulated after challenge infection; therefore, these data are not presented.

Statistics

Statistical significance was determined by Student's *t*-test, and significance was determined with p values < 0.05. All experiments were repeated two or more times with similar results.

Results

An early Th1-type cytokine mRNA response is induced in the lungs of immunized mice after infection

Mice were vaccinated with 50,000-rad-irradiated cercariae and challenged at 5 wk postimmunization, their lungs were excised for RT-PCR on days 7 to 28, and draining lymph node cells were isolated for measurement of Ag-induced recall responses on day 22 postchallenge. Although, overall, we observed a mixed Th1/Th2-like pattern of cytokine mRNA expression in both naive and immunized animals after infection with unattenuated cercariae, immunized mice seemed to be primed for strong and early Th1 reactivity (Fig. 1). Thus, a notable increase in IFN-γ, IL-12 p40, IL-2, and TNF-α mRNA was observed in vaccinated animals when compared with controls (Fig. 1, *panels A* and *B*). In contrast, Th2 cytokine mRNA levels either were not significantly different in immunized vs control animals, as was observed for IL-4, or were markedly suppressed at several time points, as in the case of IL-5 (Fig. 1, *panel C*). IL-13 expression, which was elevated on day 6 in vaccinated mice, was significantly suppressed by day 14 to 18, the time of peak production in challenged controls. Although IL-1β was increased by day 10 after challenge, there was no dramatic difference observed between any of the groups. Interestingly, IL-10 did not follow the pattern of expression exhibited by the other Th2-like cytokines, but instead more closely followed the pattern observed for IFN-γ, with significant increases in IL-10 mRNA being detected in the vaccinated group at several time points.

Depletion of IFN-γ decreases resistance and Th1 cytokine production but increases the Th2 response

Vaccinated and infected animals were treated with anti-IFN-γ mAb XMG1.6 to directly assess the contribution of IFN-γ to the evolving cytokine pattern, NO synthase expression, and parasite killing. Vaccinated mice that received anti-IFN-γ mAb harbored significantly more parasites (average 50% increase, $p < 0.001$ in three experiments) than did the vaccinated and control mAb-treated group. It is interesting to note that animals depleted of IFN-γ also showed a significant reduction in IFN-γ and IL-12 p40 mRNA expression at several time points postchallenge (Fig. 1, *panel A*), and that a similar, although less dramatic, reduction in IL-2, TNF-α, and IL-10 was seen at some time points. In contrast, IL-4, IL-5, and IL-13 (Fig. 1, *panel C*) were rapidly and significantly elevated in animals depleted of IFN-γ, thereby suggesting that a switch from predominantly Th1- to Th2-like cytokine expression occurs in vaccinated mice depleted of this cytokine. No marked change was observed in IL-1β mRNA expression.

Analysis of the Ag or mitogen responsiveness of lymph node cells obtained from anti-IFN-γ-treated animals animals on day 22 postchallenge confirmed that a switch from dominant Th1 to Th2 cytokine expression occurred in the absence of IFN-γ (Fig. 2). Vaccinated animals developed a marked anamnestic IFN-γ response upon challenge infection. Lung-associated lymph node cultures that were restimulated with Ag (SWAP) or mitogen (Con A) demonstrated a marked increase in IFN-γ production (Fig. 2). This elevation was associated with depressed expression of SWAP-specific IL-5 when compared with challenged control animals. As was observed with mRNA levels in the lung, depletion of IFN-γ blocked subsequent IFN-γ responses to both SWAP or Con A. In addition, depletion of IFN-γ restored SWAP-induced IL-5 production to the levels seen in challenged control animals.

Serum IgE levels in vaccinated animals during the 4 wk after challenge infection reflected Th2 cytokine mRNA and protein expression in the lungs (Fig. 3). Serum IgE levels were elevated earlier in animals vaccinated and challenged when compared with controls, peaking on day 14 and declining thereafter. Nevertheless, mice depleted of IFN-γ demonstrated dramatically increased serum IgE,

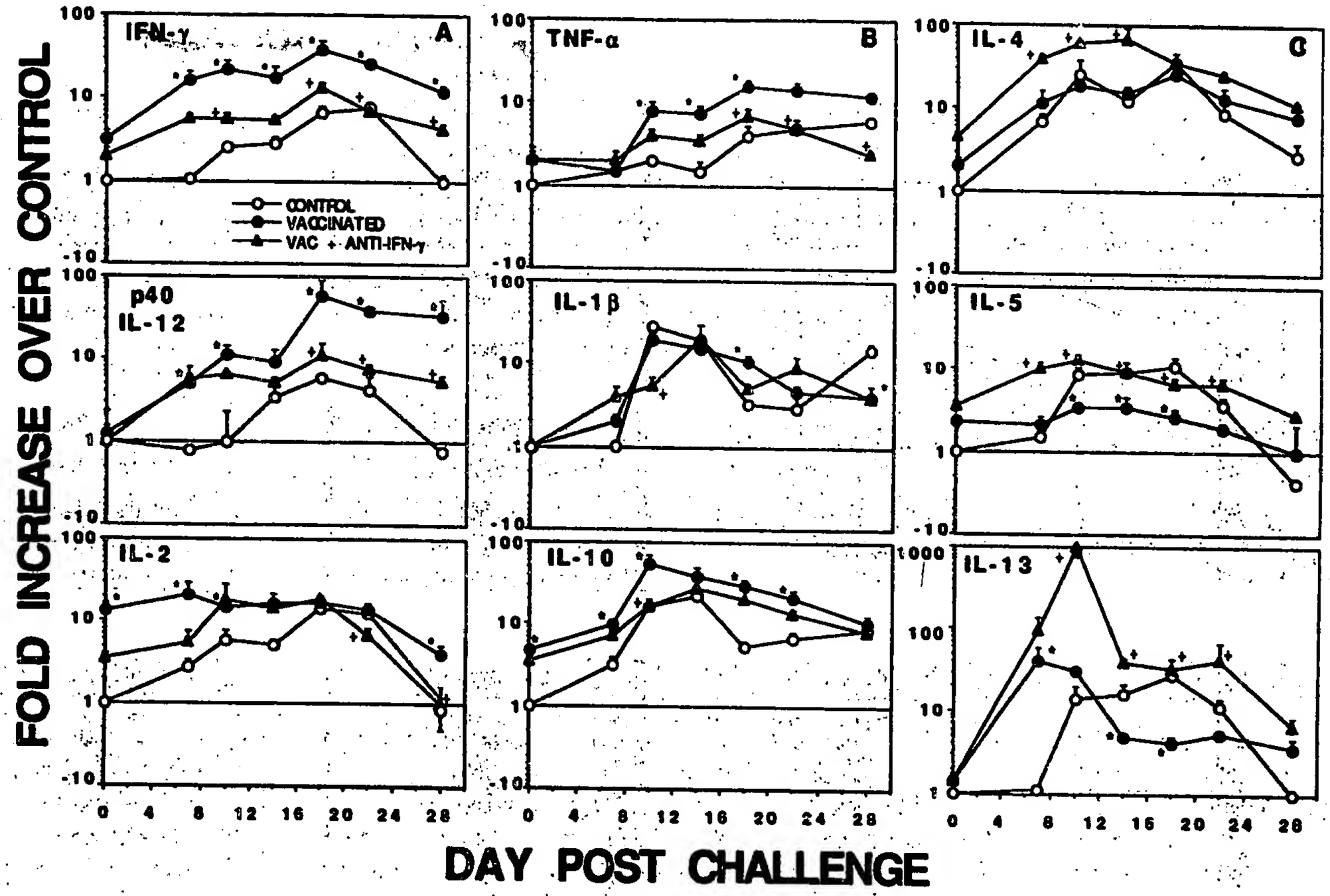


FIGURE 1. The pattern of cytokine mRNA expression in the lungs of vaccinated and control mice after challenge infection. Control (open circles) and vaccinated mice were treated either with XMG1.6 (filled triangles) to neutralize IFN-γ or with control mAb GL113 (filled circles), as described in *Materials and Methods*. All animals were challenged percutaneously with 500 unattenuated cercariae, four animals/group were killed on days 7 through 28, and total lung RNA was isolated for RT-PCR analysis. Increases in cytokine-specific mRNAs were calculated from standard curves and are reported as fold increases in mRNA expression over that observed in untreated (unchallenged controls) mouse lungs ± SE. Changes in mRNA expression for several Th1-associated (*panel A*), inflammatory/immunomodulatory (*panel B*), or Th2 cytokines (*panel C*) were determined. * indicates that the fold increase observed in vaccinated animals (filled circles) is significantly ($p < 0.05$) different from the expression in challenged controls (open circles) at that time point. † indicates that the cytokine mRNA level observed in vaccinated and GL113-treated mice was significantly different from that in the vaccinated and XMG1.6-treated group.

reaching levels nearly 3 times greater than those seen in the vaccinated and control mAb-treated animals.

Peak NO synthase induction corresponds with elevated IFN-γ expression

Macrophages and endothelial cells stimulated with combinations of the cytokines IFN-γ, TNF-α, IL-2, and IL-1 have been shown to produce elevated levels of NO in vitro through the cytokine-induced activity of NO synthase (13, 14). To determine whether a similar increase in NO production occurs in vivo, pulmonary NO synthase mRNA levels were measured after challenge infection. Vaccinated animals demonstrated a twofold to threefold increase in NO synthase mRNA in the lungs at day 7 postchallenge. Maximal increases (8.5-fold) were reached by day 18, and then levels decreased by days 22 to 28 (Fig. 4). Control animals did not display significant increases until several days later, reaching maximal levels (3.5-fold) by days 14 to 18 and returning to, or even falling below, base line levels by day 28. In vaccinated animals depleted of IFN-γ, NO synthase was elevated only at a single time point, demonstrating no change over base line levels at any other time, which suggests an important role for IFN-γ in promoting NO synthase expression.

In other experiments, naive and vaccinated animals were challenged i.v. with mechanically transformed cercariae. Under these conditions, the parasites reach the lung simultaneously, thereby allowing a synchronized cytokine response so that more detailed analyses of the evolving cytokine patterns could be detected. IFN-γ and NO synthase mRNA levels were evaluated 1 to 14 days postchallenge. In these experiments, IFN-γ mRNA was

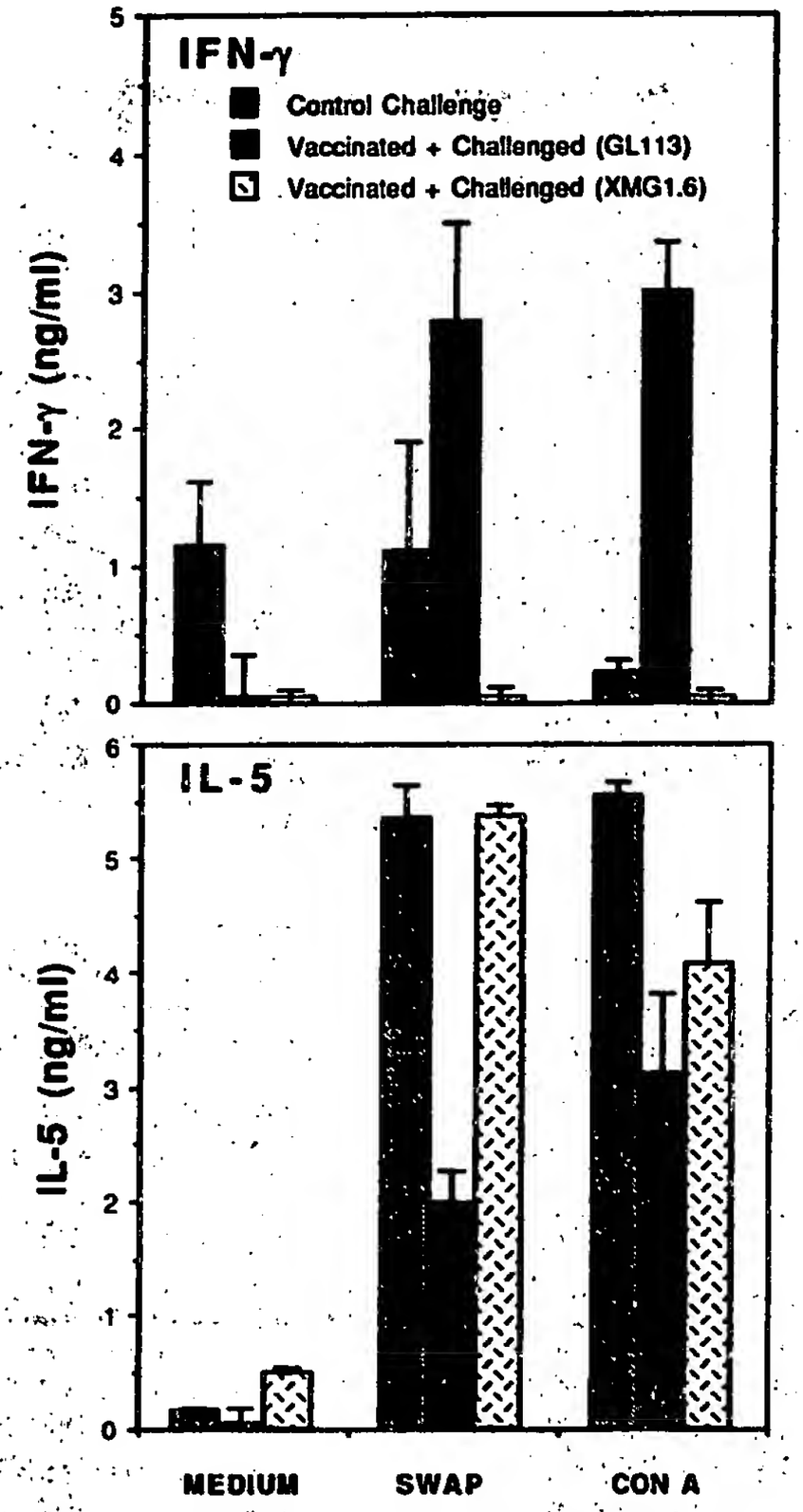


FIGURE 2. Neutralization of IFN-γ restores lymph node SWAP-specific IL-5 secretion while suppressing IFN-γ. Lung-associated lymph nodes were isolated from the animals described in Figure 1 on day 22 postchallenge and restimulated with medium alone, SWAP, or Con A. Culture supernatants were isolated at 72 h and assayed by ELISA for the production of IFN-γ and IL-5, as indicated. The reactivities of lymph node cells from four individual mice are illustrated and are reported in nanograms per milliliter ± SD.

rapidly increased in both control and vaccinated animals; however, the response in vaccinated animals peaked by day 1, at levels 10-fold higher than those in unvaccinated controls (Fig. 5). Peak expression in controls was not only lower, but occurred one day later than in immunized mice, suggesting an anamnestic IFN-γ response in the immunized group. NO synthase mRNA expression followed similar kinetics. Although peak expression in both groups occurred by day 2, vaccinated animals demonstrated a more than fourfold greater increase in NO synthase expression than did challenged controls.

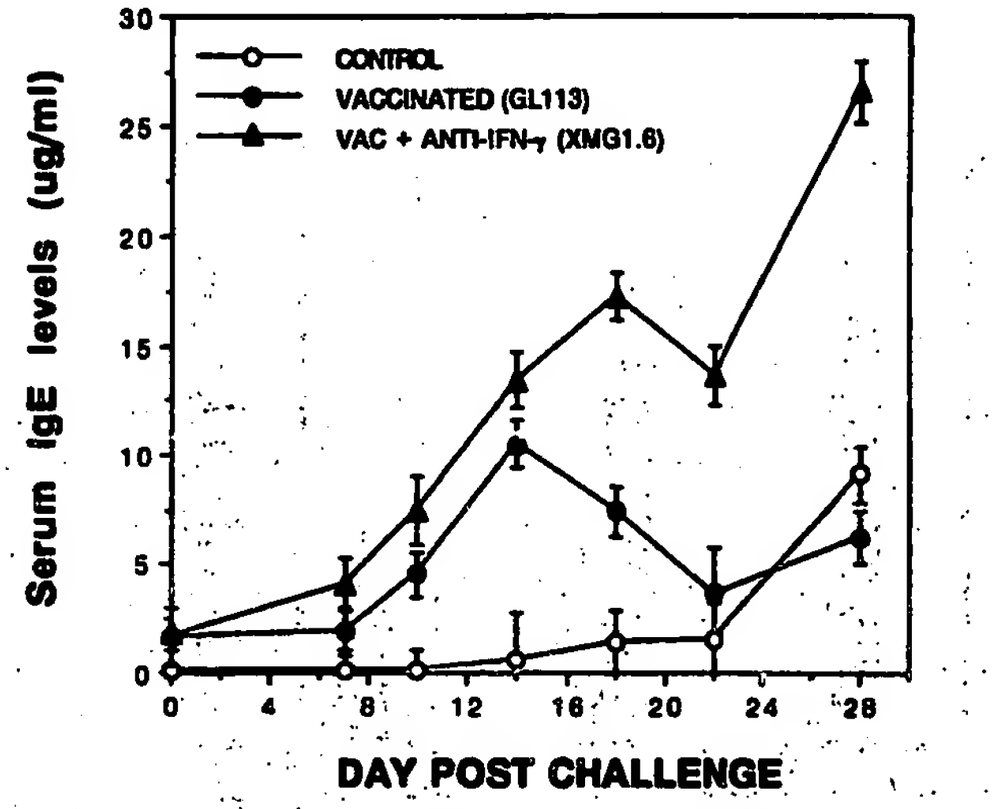


FIGURE 3. Serum IgE levels are elevated in vaccinated and challenged mice and further enhanced as a consequence of IFN-γ depletion. The animals described in Figure 1 were bled on the days indicated, and total serum IgE levels were assessed for three to four individual mice at each time point, as described in *Materials and Methods*. The data are reported in micrograms per milliliter ± SE.

NO synthase expression is localized to inflammatory foci in animals vaccinated and challenged i.v. with schistosomula

To confirm and extend the mRNA studies, we stained for NO synthase in histologic sections prepared from vaccinated animals challenged i.v. with schistosomula. The peak time point of NO synthase mRNA expression (Fig. 5) was chosen for these studies. As seen in Figure 6*A*, nearly all of the inflammatory foci surrounding challenge parasites exhibited positive staining (arrows) for NO synthase. In contrast, lung sections stained with control serum demonstrated no staining (Fig. 6*B*).

Blocking NO synthase activity in vivo increases worm burdens in both control and vaccinated animals

To assess the contribution of NO in parasite attrition, after parasite challenge, control and vaccinated animals were treated twice daily with AMG to block production of NO. In *panel A* of Figure 7, immunized animals were vaccinated with 500 irradiated cercariae, whereas in *panel B*, mice were vaccinated with only 50 irradiated cercariae to reduce the level of protection and improve the possibility of seeing an effect of the inhibitor. Interestingly, all animals treated with the inhibitor demonstrated a 12 to 22% increase in worm recovery over that observed in animals treated with saline alone. In both sets of experiments, vaccinated mice treated with AMG had significantly higher ($p < 0.05$) worm burdens than did PBS-treated vaccinated animals, with the most dramatic increase being observed

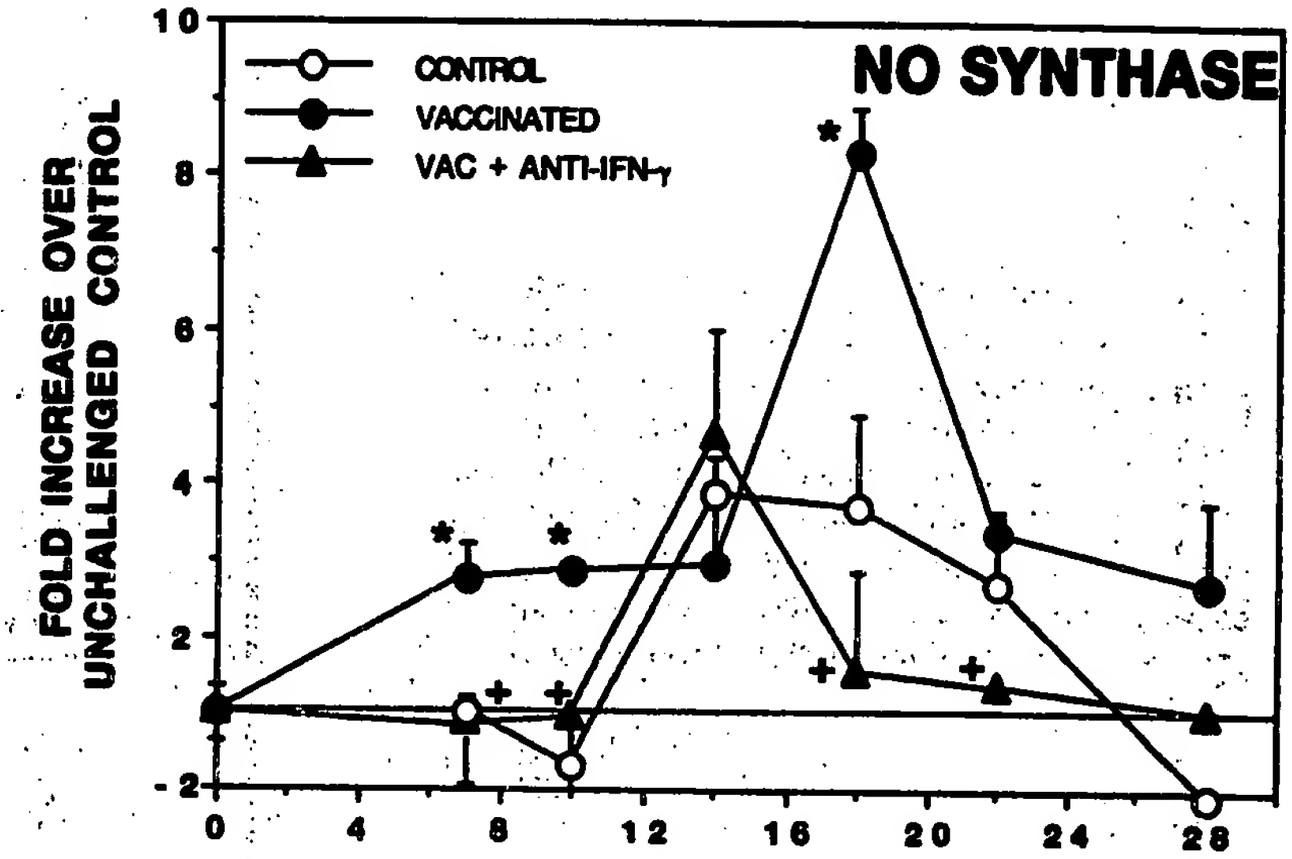


FIGURE 4. NO synthase mRNA expression is increased in the lungs of vaccinated and challenged mice and suppressed in animals that have been depleted of IFN-γ. Lung NO synthase mRNA levels were assessed by RT-PCR with use of the cDNAs described in the legend to Figure 1. The data are reported as fold increases over unchallenged control mRNA levels ± SE.

in animals vaccinated with the low dose of irradiated cercariae.

Discussion

Several in vivo studies have suggested that the lung is the primary site for immune elimination of challenge *S. mansoni* larvae in mice immunized with radiation-attenuated cercariae (34–38). This process of vaccine-induced worm attrition is associated with an anamnestic T cell response in the lungs, production of IFN-γ by cells recovered from bronchoalveolar lavage, and mononuclear cell-rich inflammatory reactions surrounding challenge parasites (18, 39, 40). We have shown that macrophages (11) and endothelial cells (13) become activated to produce toxic NO and to kill schistosomula after in vitro stimulation by combinations of the cytokines IFN-γ, TNF-α, IL-1, and IL-2, and we hypothesized that this response may form the basis of protective immunity in this model (11). To determine whether the same mechanisms may operate in vivo, we have, by using RT-PCR, analyzed the cytokine mRNA response in the lungs of mice vaccinated and challenged with cercariae.

In general, the data presented here regarding the evolving lung cytokine mRNA response in vaccinated mice support previous observations that suggested a dominance of Th1-like cytokine expression in animals vaccinated with attenuated cercariae, in contrast with a dominance of Th2 cytokine expression in animals with patent *S. mansoni* infection (11). We found an earlier and stronger IFN-γ and IL-2 mRNA response in the lungs of immunized animals when compared with challenged controls (Fig 1). In contrast with previous studies (18), Th2-like cytokine mRNAs also were detected, although often at levels similar to, or even lower than, those in controls. In the case of IL-10,

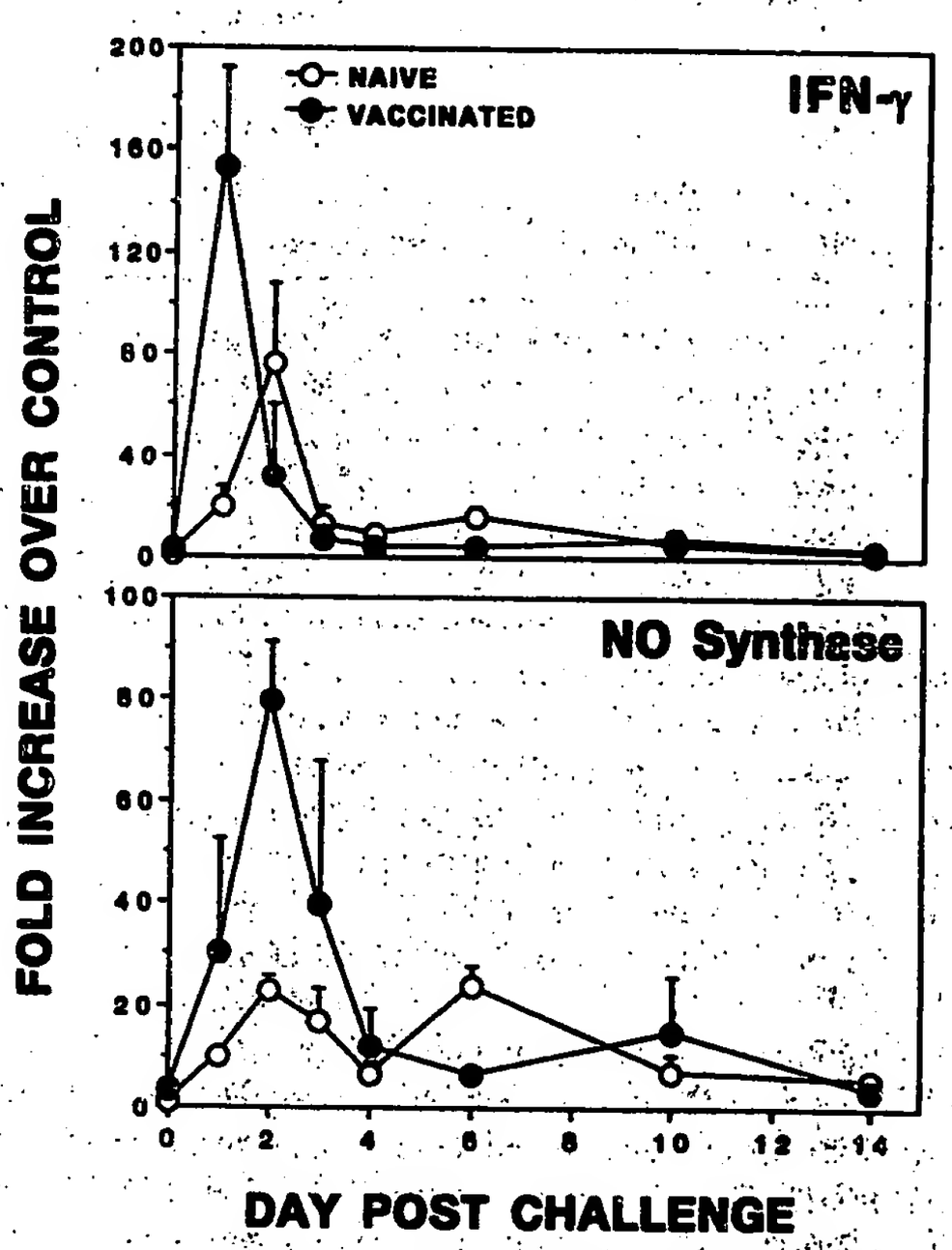


FIGURE 5. Expression of NO synthase and IFN-γ mRNA in vaccinated and control mice challenged i.v. with schistosomula. Naive (open circles) or vaccinated (filled circles) mice were challenged i.v. with 500 mechanically transformed schistosomula. Animals were killed on the days indicated, and total lung RNA was assayed by RT-PCR for IFN-γ and NO synthase. The data are reported as fold increases over unchallenged control mRNA levels ± SE.

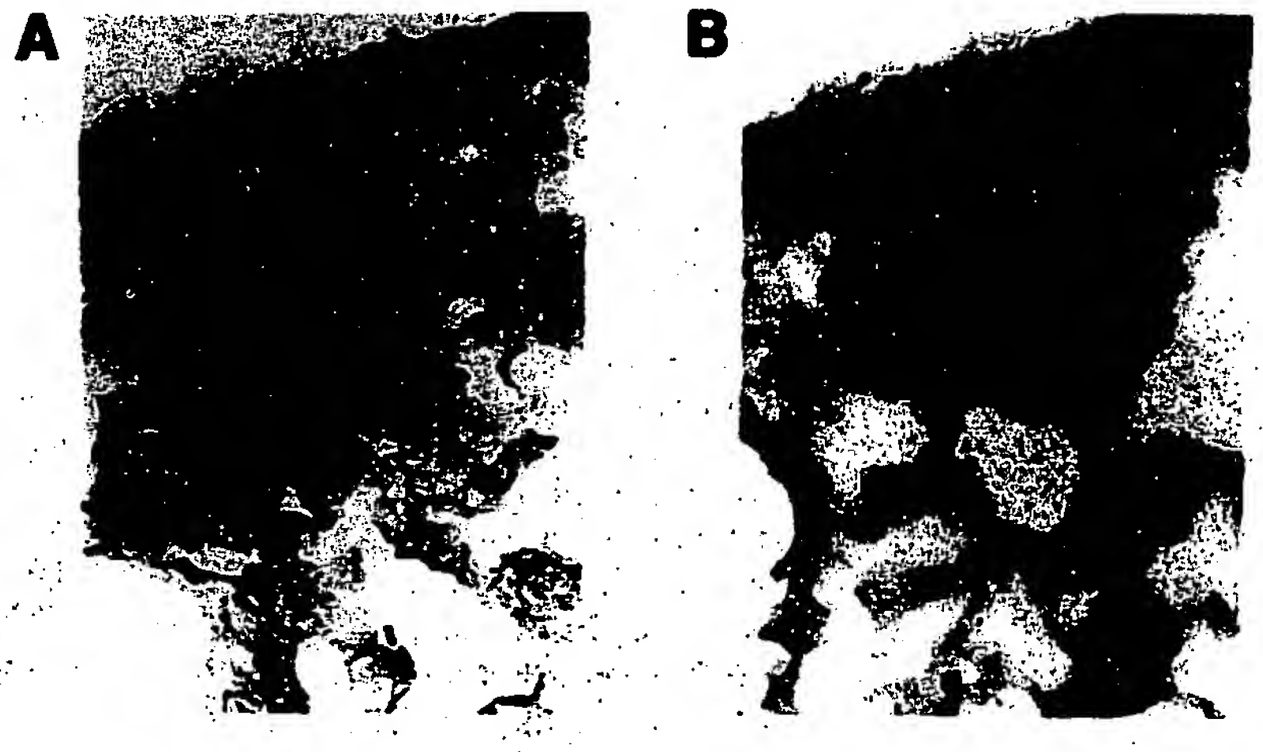


FIGURE 6. Pulmonary NO synthase expression is localized to the inflammatory foci surrounding the schistosomula. A portion of the right lung was obtained from the mice described in the legend to Figure 5 and analyzed by immunohistochemistry for expression of NO synthase by using a polyclonal serum specific for the enzyme (*panel A*). A control serum was used for the serial section stained in *panel B*. The section shows a representative inflammatory focus observed on day 2 in vaccinated animals challenged i.v. with mechanically transformed schistosomula. The arrows indicate the areas that are positive for NO synthase.

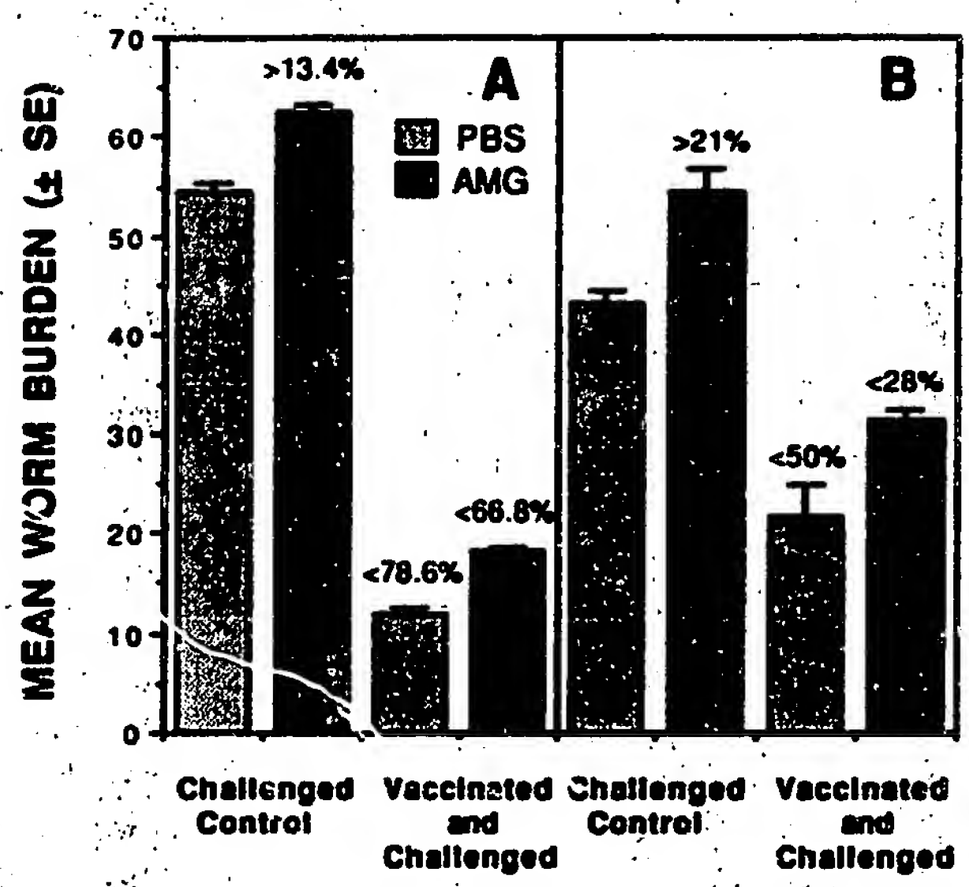


FIGURE 7. The inhibition of NO synthase activity in vivo with AMG significantly increases worm recovery. C57BL/6 mice were vaccinated with 500 (*panel A*) or 50 (*panel B*) 50,000-rad-irradiated cercariae. Five weeks later, 10 animals/group were challenged percutaneously with 120 non-attenuated cercariae, and worm recovery was assessed 6 wk later. Animals were treated twice daily with saline (shaded bars) or AMG (filled bars) for 4 wk after challenge, as described in *Materials and Methods*. The AMG groups in both experiments all were significantly ($p < 0.05$) different from their respective controls. The number shown above each bar represents the percent change in worm numbers vs the control challenged and saline-treated group.

however, mRNA levels seemed to be even higher in immunized vs control challenged mice (Fig. 1, *panel B*). This co-expression of IL-10 and IFN-γ has now been frequently observed in studies of cytokine expression in vivo (32, 41, 42).

The observation that IFN-γ is substantially elevated in vaccinated and challenged mice implies that this cytokine is likely to play a major role in the elimination of parasites. This finding has been confirmed by cytokine depletion studies in which the neutralization of IFN-γ, but not IL-4 or IL-5, at the time of challenge reduced protection (9). More recent studies suggested that maximal neutralization of IFN-γ may abrogate immunity by as much as 90% (10). In those studies, neutralization of IFN-γ, while decreasing protection, increased the recovery of bronchoalveolar lavage cells and also increased the size of lesions surrounding the migrating parasites (10). This increase was suggested to be caused by increased numbers of eosinophils and multinucleated giant cells within the inflammatory foci. In this study, we observed similar increases in eosinophils and giant cells, as well as marked increases in IL-4 and IL-5 expression, in the lungs of mice depleted of IFN-γ (data not shown). Therefore, the increased numbers of these cells are likely the result of increased Th2 cytokine secretion, because IL-4 has been shown to be important in the formation of giant cells (43), and IL-5 is critical for the differentiation of eosinophils (9).

Together, these and previous observations suggest that an increased ratio of Th2- to Th1-like cytokine expression has occurred in immunized mice depleted of IFN-γ. This altered pattern was observed both in the lung at the mRNA level (Fig. 1) and in the draining lymph nodes after in vitro stimulation with SWAP (Fig. 2). Additional evidence for a switch in the Th cell phenotype was indicated by the serum IgE levels (Fig. 3). The marked increase in serum IgE in anti-IFN-γ-treated mice was likely the result of enhanced IL-4 secretion and simultaneous loss of the inhibitory effect of IFN-γ on B cell switching to IgE production (44). Thus, these data are consistent with the hypothesis that IFN-γ plays a major role in promoting Th1 cell differentiation while suppressing that of Th2 (45, 46).

Previous studies have shown that IFN-γ is a major cytokine involved in the activation of macrophages and endothelial cells for larvicidal activity, although different combinations of the cytokines TNF-α, IL-1, IL-2, and IL-6 have been found to serve as costimulatory factors (13, 14). Although IFN-γ mRNA was markedly increased at nearly

all time points in the lungs of vaccinated vs challenged controls, we also noted significant increases in IL-2, TNF-α, and IL-1β (Fig. 1). Together, these data demonstrate that a cytokine microenvironment that is conducive to the activation of macrophages and endothelial cells is induced in the lungs of immunized mice, which suggests that the effector mechanisms described previously in vitro may also operate in vivo.

Studies of the effector mechanism of in vitro larvicidal activity have indicated that cytokine activation induces the arginine-dependent production of reactive NO, which kills the parasites (16, 47). In our in vivo studies, we found elevated NO synthase mRNA expression in the lungs of all challenged mice (Fig. 4). Interestingly, peak NO synthase mRNA expression occurred at a time when elimination of migrating larvae is believed to occur (17, 37, 48) and levels of IFN-γ mRNA have reached a maximum (Fig. 1). Depletion studies indicated that the increase in NO was highly dependent on this cytokine (Fig. 4). In addition, in mice injected i.v. with schistosomula, similar kinetic patterns of IFN-γ and NO synthase mRNA expression were observed (Fig. 5). Together, these data provide the first in vivo evidence for cytokine regulation by a parasite vaccine of NO synthase, the enzyme responsible for the production of this crucial immune effector molecule.

Investigations of the fate of challenge parasites in vaccinated mice indicate that the larvae passing through the lungs become trapped in inflammatory reactions that are histologically reminiscent of delayed-type hypersensitivity responses (35, 36), a Th1-associated phenomenon (49). It has been suggested that this delayed-type hypersensitivity reaction effectively traps the larvae in the lungs, where they eventually die (17). Our studies confirm that NO synthase is induced in the lungs of challenged animals and demonstrate that the expression of the enzyme is localized primarily to the inflammatory foci surrounding the schistosomula (Fig. 6). It is possible that the local production of NO is directly toxic to the parasites as they migrate through the lungs, as has been shown in vitro (16). In addition, pulmonary inflammation, which impedes their intravascular migration, may effectively increase larval exposure to NO, thereby creating a toxic microenvironment from which they must escape, which encourages their deflection into the alveoli (37, 38, 50). The results presented here (Fig. 7), which show that treatment of vaccinated mice with AMG at the time of challenge increased the worm burden by 32 to 33%, certainly suggest an in vivo role for NO in protective immunity. The fact that resistance was not totally ablated may simply indicate that AMG treatment did not completely eliminate NO production in vivo or, more likely, reflects a role for other immune mechanisms or mediators. Surprisingly, control mice also demonstrated an average 18.3% increase in worm numbers when they were treated with AMG at the time of challenge, which suggests an innate, non-vaccine-induced role for NO in parasite elimination as well. This finding may be related to an earlier observation that a significant cell-mediated immune response (as suggested by increased Ag-specific lymphocyte proliferation) can be detected within a few days after schistosome infection of naive mice (51).

Recent studies have shown that IFN-γ activation of macrophages for NO-mediated killing is inhibitable by IL-10 and IL-4 (14, 52). Although these cytokines were individually active at high levels, very low concentrations of both mediators inhibited macrophage larvicidal activity and NO production in a synergistic fashion (52). Likewise, a recently described cytokine, IL-13, which has both structural and functional homology with IL-4, also has been found to modulate macrophage function (53) and suppress NO production (54). Although IFN-γ was markedly elevated in vaccinated and challenged mice, we also detected significant increases in IL-4, IL-10, and IL-13 (Figs. 1 and 2). Moreover, depletion of IFN-γ, while increasing worm burdens, resulted in even greater IL-4 and IL-13 lung mRNA levels (Fig. 2). The fact that these potent downregulatory cytokines are induced in the lungs of immunized mice may explain why the attenuated vaccine fails to confer complete protection. Because neutralization of IL-4 alone previously failed to affect vaccine-induced immunity (9), it may be necessary to neutralize several inhibitory cytokines to observe a significant increase in protection.

In conclusion, these studies confirm and extend previous studies that have suggested a critical role for IFN-γ in the protection of mice vaccinated with attenuated cercariae and provide in vivo evidence for a role for NO in parasite attrition. Recently, IL-12 has been identified as a major regulatory cytokine that influences the production of IFN-γ by several cell types (55). It is interesting to note that, in vaccinated mice, we observed marked increases in IL-12 p40 mRNA (Fig. 1), which were elevated most significantly within a few days after parasite challenge. Therefore, one focus of our current work is to evaluate the role of endogenous IL-12 in the irradiated vaccine model. In addition, because of the published suppressive effects of rIL-12 on parasite-induced Th2 responses (32, 56, 57), experiments have been initiated to determine whether exogenous rIL-12, when administered together with irradiated cercariae, can suppress Th2-like cytokine production in the lungs and, thereby, increase Th1-associated immunity induced by schistosome vaccines. Indeed, our preliminary data suggest that rIL-12 can markedly enhance immunity induced by the attenuated cercariae vaccine.

Acknowledgments

We thank Fred Finkelman and Ramona Geffrics for determining serum IgE levels and Allen Cheever and Dragana Jankovic for their helpful advice and for critically reviewing the manuscript. We also acknowledge the excellent technical assistance of Sara Hieny.

References

1. Cheever, A. W. 1993. Schistosomiasis: infection versus disease, hypersensitivity and immunity. *Am. J. Pathol. 142:699.*
2. James, S. L., and D. L. Boros. 1994. Immune effector role of macrophages in experimental schistosomiasis mansoni. *Immunol. Ser. 60:461.*
3. Damian, R. T. 1984. Immunity in schistosomiasis: a holistic view. *Contemp. Top. Immunobiol. 12:359.*
4. Sher, A., S. Hieny, S. L. James, and R. Asofsky. 1982. Mechanisms of protective immunity against *Schistosoma mansoni* in mice vaccinated with irradiated cercariae. II. Analysis of immunity in hosts deficient in T lymphocytes, B lymphocytes, and complement. *J. Immunol. 128:1880.*
5. Correa-Oliveira, R., and A. Sher. 1985. Defective immunoglobulin M responses to vaccination or infection with *Schistosoma mansoni* in *xid* mice. *Infect. Immun. 50:409.*
6. Sher, A., R. Correa-Oliveira, S. Hieny, and R. Hussain. 1983. Mechanisms of protective immunity against *Schistosoma mansoni* in mice vaccinated with irradiated cercariae. IV. Analysis of the role of IgE antibodies and mast cells. *J. Immunol. 131:1460.*
7. Vignali, D. A., P. Crocker, Q. D. Bickle, S. Cobbold, H. Waldmann, and M. G. Taylor. 1989. A role for $CD4^+$ but not $CD8^+$ T cells in immunity to *Schistosoma mansoni* induced by 20-kRad-irradiated and Ro 11–3128-terminated infections. *Immunology 67:466.*
8. Kelly, E., and D. G. Colley. 1988. In vivo effects of monoclonal anti-L3T4 antibody on immune responsiveness of mice infected with *Schistosoma mansoni*: reduction of irradiated cercariae-induced resistance. *J. Immunol. 140:2737.*
9. Sher, A., R. L. Coffman, S. Hieny, and A. W. Cheever. 1990. Ablation of eosinophil and IgE responses with anti-IL-5 or anti-IL-4 antibodies fails to affect immunity against *Schistosoma mansoni* in the mouse. *J. Immunol. 145:3911.*
10. Smythies, L. E., P. S. Coulson, and R. A. Wilson. 1992. Monoclonal antibody to IFN-γ modifies pulmonary inflammatory responses and abrogates immunity to *Schistosoma mansoni* in mice vaccinated with attenuated cercariae. *J. Immunol. 149:3654.*
11. James, S. L., and C. Nacy. 1993. Effector functions of activated macrophages against parasites. *Curr. Opin. Immunol. 5:518.*
12. James, S. L., and A. Sher. 1983. Mechanisms of protective immunity against *Schistosoma mansoni* in mice vaccinated with irradiated cercariae. III. Identification of a mouse strain, P/N, that fails to respond to vaccination. *Parasite Immunol. 5:567.*
13. Oswald, I. P., I. Eltoum, T. A. Wynn, B. Schwartz, P. Caspar, D. Paulin, A. Sher, and S. L. James. 1994. Endothelial cells are activated by cytokine treatment to kill and intravascular parasite, *Schistosoma mansoni*, through the production of nitric oxide. *Proc. Natl. Acad. Sci. USA 91:999.*
14. Oswald, I. P., T. A. Wynn, A. Sher, and S. L. James. 1992. Interleukin-10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor α required as a costimulatory factor for interferon-γ-induced activation. *Proc. Natl. Acad. Sci. USA 89:8676.*
15. Gazzinelli, R. T., I. P. Oswald, S. L. James, and A. Sher. 1992. IL-10 inhibits parasite killing and nitrogen oxide production by IFN-γ-activated macrophages. *J. Immunol. 148:1792.*
16. James, S. L., and J. A. Glaven. 1989. Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. *J. Immunol. 143:4208.*
17. Wilson, R. A., P. S. Coulson, and B. Dixon. 1986. Migration of the schistosomula of *Schistosoma mansoni* in mice vaccinated with radiation-attenuated cercariae, and normal mice: an attempt to identify the timing and site of parasite death. *Parasitology 92:101.*
18. Smythies, L. E., R. M. Pemberton, P. S. Coulson, A. P. Mountford, and R. A. Wilson. 1992. T cell-derived cytokines associated with pulmonary immune mechanisms in mice vaccinated with irradiated cercariae of *Schistosoma mansoni*. *J. Immunol. 148:1512.*
19. Lazdins, J. R., M. J. Stein, J. R. David, and A. Sher. 1982. *Schistosoma mansoni*: rapid isolation and purification of schistosomula of different developmental stages by centrifugation on discontinuous density gradients of Percoll. *Exp. Parasitol. 53:39.*
20. James, S. L., R. Correa-Oliveira, and E. Leonard. 1984. Defective vaccine-induced immunity to *Schistosoma mansoni* in P strain mice. II. Analysis of cellular responses. *J. Immunol. 133:1587.*
21. Minard, P., D. A. Dean, R. Jacobson, W. Vannier, and K. D. Murrell. 1978. Immunization of mice with cobalt-60-irradiated *Schistosoma mansoni* cercariae. *Am. J. Trop. Med. Hyg. 27:76.*
22. Caulada-Benedetti, Z., F. Al-Zamel, A. Sher, and S. James. 1991. Comparison of Th1- and Th2-associated immune reactivities stimulated by single versus multiple vaccination of mice with irradiated *Schistosoma mansoni* cercariae. *J. Immunol. 146:1655.*
23. von Lichtenberg, F., A. Sher, and S. McIntyre. 1977. A lung model of schistosome immunity in mice. *Am. J. Pathol. 87:105.*
24. Grzych, J.-M., E. Pearce, A. Cheever, Z. A. Caulada, P. Caspar, S. Heiny, F. Lewis, and A. Sher. 1991. Egg deposition is the major stimulus for production of Th2 cytokines in murine schistosomiasis mansoni. *J. Immunol. 146:1322.*
25. Curry, R. C., P. A. Keiner, and G. L. Spitalny. 1987. A sensitive immunochemical assay for biologically active Mu IFN-γ. *J. Immunol. Methods 104:137.*
26. Schumacher, J. H., A. O'Garra, B. Schrader, A. van Kimmenade, M. W. Bond, T. R. Mosmann, and R. L. Coffman. 1988. The characterization of four monoclonal antibodies specific for mouse IL-5 and development of mouse and human IL-5 enzyme-linked immunosorbent assays. *J. Immunol. 141:1576.*
27. Finkelman, F. D., C. M. Snapper, J. D. Mountz, and I. M. Katona. 1987. Polyclonal activation of the murine immune system by an antibody to IgD. IX. Induction of a polyclonal IgE response. *J. Immunol. 138:2826.*
28. Beckerman, K. P., H. W. Rogers, J. A. Corbett, M. L. McDaniel, and E. R. Unanue. 1993. Release of nitric oxide during the T cell-independent pathway of macrophage activation. *J. Immunol. 150:888.*
29. Wynn, T. A., I. Eltoum, A. W. Cheever, F. A. Lewis, W. C. Gause, and A. Sher. 1993. Analysis of cytokine mRNA expression during primary granuloma formation induced by eggs of *Schistosoma mansoni*. *J. Immunol. 151:1430.*
30. Lowenstein, C. J., C. S. Glatt, D. S. Bredt, and S. H. Snyder. 1992. Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc. Natl. Acad. Sci. USA 89:6711.*
31. Svetic, A., F. D. Finkelman, Y. C. Jian, C. W. Dieffenback, D. E. Scott, K. F. McCarthy, A. D. Steinberg, and W. C. Gause. 1991. Cytokine gene expression after in vivo primary immunization with goat antibody to mouse IgD antibody. *J. Immunol. 147:2391.*
32. Wynn, T. A., I. Eltoum, I. P. Oswald, A. W. Cheever, and A. Sher. 1994. Endogenous IL-12 regulates granuloma formation induced by eggs of *Schistosoma mansoni* and exogenous IL-12 both inhibits and prophylactically immunizes against egg pathology. *J. Exp. Med. 179:1551.*
33. Gazzinelli, R. T., I. Eltoum, T. A. Wynn, and A. Sher. 1993. Acute cerebral toxoplasmosis is induced by in vivo neutralization of TNF-α and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. *J. Immunol. 151:3672.*
34. Mangold, B. L., and D. L. Dean. 1984. The migration and survival of gamma-irradiated *Schistosoma mansoni* larvae and the duration of host-parasite contact in relation to the induction of resistance in mice. *Parasitology 88:249.*
35. Wilson, R. A., and P. S. Coulson. 1989. Lung-phase immunity to schistosomes: a new perspective on an old problem. *Parasitol. Today 5:274.*
36. Coulson, P. S., and R. A. Wilson. 1988. An examination of the mechanisms of pulmonary phase resistance to *Schistosoma mansoni* in vaccinated mice. *Am. J. Trop. Med. Hyg. 38:529.*
37. Dean, D. A., and B. L. Mangold. 1992. Evidence that both normal and immune elimination of *Schistosoma mansoni* take place at the lung stage of migration prior to parasite death. *Am. J. Trop. Med. Hyg. 47:238.*
38. Kassim, O. O., D. A. Dean, B. L. Mangold, and F. von Lichtenberg. 1992. Combined microautoradiographic and histopathologic analysis of the fate of challenge *Schistosoma mansoni* schistosomula in mice

immunized with irradiated cercariae. *Am. J. Trop. Med. Hyg. 47:231.*

39. Crabtree, J. E., and R. A. Wilson. 1986. The role of pulmonary cellular reactions in the resistance of vaccinated mice to *Schistosoma mansoni*. *Parasite Immunol. 8:265.*
40. Kambara, T., and R. A. Wilson. 1990. In situ pulmonary responses of T cell and macrophage subpopulations to a challenge infection in mice vaccinated with irradiated cercariae of *Schistosoma mansoni*. *J. Parasitol. 76:365.*
41. Karp, C. L., S. H. El-Safi, T. A. Wynn, M. M. H. Satti, M. Kordofani, M. Hag-ali, F. A. Neva, and D. L. Sacks. 1993. In vivo cytokine profiles in patients with kala azar: marked elevation of both IL-10 and IFN-γ. *J. Clin. Invest. 91:1644.*
42. Morris, S. C., K. D. Madden, J. J. Adamovicz, W. C. Gause, B. R. Hubbard, M. K. Gately, and F. D. Finkelman. 1994. Effects of IL-12 on in vivo cytokine gene expression and Ig isotype selection. *J. Immunol. 152:1047.*
43. McInnes, A., and L. Rennick. 1988. IL-4 induces cultured monocytes/macrophages to form giant multinucleated cells. *J. Exp. Med. 167:598.*
44. Finkelman, F. D., J. Holmes, I. M. Katona, J. F. Urban, M. P. Beckmann, L. S. Park, K. A. Schooley, R. L. Coffman, T. R. Mosmann, and W. E. Paul. 1990. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu. Rev. Immunol. 8:303.*
45. Gajewski, T. F., and F. W. Fitch. 1988. Anti-proliferative effect of IFN-γ in immune regulation. I. IFN-γ inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J. Immunol. 140:4245.*
46. Scott, P. 1991. IFN-γ modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *J. Immunol. 147:3149.*
47. Oswald, I. P., T. A. Wynn, A. Sher, and S. L. James. 1994. NO as an effector molecule of parasite killing: modulation of its synthesis by cytokines. *Comp. Biochem. Physiol. 108C:11.*
48. Pearce, E. J., and S. L. James. 1986. Post-lung-stage schistosomula of *Schistosoma mansoni* exhibit transient susceptibility to macrophage-mediated cytotoxicity in vitro that may relate to late phase killing in vivo. *Parasite Immunol. 8:513.*
49. Cher, D. J., and T. R. Mosmann. 1987. Two types of murine helper cell clone. II. Delayed-type hypersensitivity is mediated by Th1 clones. *J. Immunol. 138:3688.*
50. Menson, E. N., and R. A. Wilson. 1990. Lung-phase immunity to *Schistosoma mansoni*: definition of alveolar macrophage phenotypes after vaccination and challenge of mice. *Parasite Immunol. 12:353.*
51. Correa-Oliveira, R., A. Sher, and S. L. James. 1984. Mechanisms of protective immunity against *Schistosoma mansoni* infection in mice vaccinated with irradiated cercariae. *Am. J. Trop. Med. Hyg. 33:261.*
52. Oswald, I. P., R. T. Gazinelli, A. Sher, and S. L. James. 1992. IL-10 synergizes with IL-4 and transforming growth factor-β to inhibit macrophage cytotoxic activity. *J. Immunol. 148:3578.*
53. Zurawski, G., and J. E. de Vries. 1994. Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. *Immunol. Today 15:19.*
54. Doherty, T. M., R. Kastelein, S. Memon, S. Andrade, and R. L. Coffmann. 1993. Modulation of murine macrophage function by IL-13. *J. Immunol. 151:7151.*
55. Chan, S. H., B. Perussia, J. W. Gupta, M. Kobayashi, M. Pospisil, H. A. Young, S. F. Wolf, D. Young, S. C. Clark, and G. Trinchieri. 1991. Induction of interferon γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J. Exp. Med. 173:869.*
56. Afonso, L. C. C., T. M. Scharton, L. Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott. 1994. The adjuvant effect of interleukin 12 in a vaccine against *Leishmania major*. *Science 263:235.*
57. Finkelman, F. D., K. Madden, A. W. Cheever, I. M. Katona, S. C. Morris, M. Gately, B. R. Hubbard, W. C. Gause, and J. F. Urban, Jr. 1994. Effects of interleukin-12 on immune responses and host protection in mice infected with intestinal nematode parasites. *J. Exp. Med. 179:1563.*

STIC-ILL

From: Prasad, Sarada
Sent: Tuesday, March 06, 2001 5:33 PM
To: STIC-ILL

Please fetch me the following full text citations for the case # 09,395,038

1. Philosophical transactions of the roayal Society of London B Biological Sciences (Sept. 29, 1997) vol. 352, No. 1359, pages 1311-1315.(cytokines and nitric oxide as effector molecules against parasitic infections.
2. J Immunol. 1994, vol.153, No.11, pages 5200-5209. (Elevated expression of Th1 cytokines and nitric oxide synthase in the lungs of vaccinated mice after challenge infection with schistosoma mansoni.)
3. Cancer immunol, Immunotherpy, 1996 jan, vol.42, No.1, pages 38-46. (Effects of N-methyl arg, an inhibitor of nitric oxide synthesis on IL-2 induced capillary leakage and anti-tumor responses innhealthy and tumor bearing mice.
4. Infection and immunity 1997, vol. 65, No. 4 1307-1312. (Expression of cytokines and inducible nitric oxuide synthase mRNA in the lungs of mice infected with cryptococcus neoformans: effects of interleukin 12).
5. Journal of general virology 1998, vol.79, No. 4, pages 825-830 (Mice lacking inducible niric oxide synthase are more susceptible to herpers simplex virus infection despite enhanced Th1 cells responses)
6. J Neuroimmunology 1998, vol.88, No. 1-2, pages 101-108. (Interferon gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons.)
7. J Experimental medicine 1996, vol.183, No. 4, pages 1447-1459. (The role of interleukin 12 and nitric oxide in the development of spontaneous auroimmune disese in mrl...mice).

thank you,

Sarada Prasad, Ph.D.
Patent Examiner
Art Unit 1646
Room CM1-8B17
Tel: 703-305-1009

ELSEVIER

Journal of Neuroimmunology 68 (1996) 101–108

Journal of Neuroimmunology

Interferon-γ induced type I nitric oxide synthase activity inhibits viral replication in neurons

Takashi Komatsu [a], Zhengbiao Bi [a,1], Carol S. Reiss [a,b,c,*]

[a] *Department of Biology, New York University, New York, NY 10003, USA*
[b] *Center for Neural Science, New York University, New York, NY 10003, USA*
[c] *Kaplan Cancer Center, New York University, New York, NY 10003, USA*

Received 19 March 1996; revised 1 April 1996; accepted 19 April 1996

Abstract

Type I NOS expression increases in OB neurons during VSV infection. Immunocytochemical staining of NB41A3 cells indicates constitutive expression of interferon (IFN)-γ receptor and type I NOS. IFN-γ treatment of NB41A3 cells increased NO production and type I NOS protein. In vitro replication of VSV, polio virus type 1, and Herpes Simplex virus type 1 (HSV-1) is significantly inhibited by IFN-γ induced type I NOS and antagonized by NOS inhibitors. In contrast, while IFN-γ treatment inhibited influenza and Sindbis virus replication, a different pathway(s) was involved. The isoform-selective NOS inhibitor, 7-nitroindazole (7NI) was used to treat mice; resulting in a 10-fold higher titer of virus in brain homogenates, and abrogated the recovery-promoting effect of interleukin-12 treatment. Thus, IFN-γ induced type I NOS activity may play an important role in host immunity against neurotropic viral infections.

Keywords: Interferon-γ; Neuronal nitric oxide synthase; Viral infection

1. Introduction

VSV is a single-stranded, negative-sense RNA virus. When experimentally applied intranasally to BALB/c mice, VSV causes an initial infection of the olfactory receptor neurons (Plakhov et al., 1995) and the olfactory bulb (OB) followed by an acute infection of the CNS (Lundh et al., 1987; Forger et al., 1991; Huneycutt et al., 1994). Viral titers peak at day 7–10 post infection when mortality occurs. Surviving mice completely clear the virus from the CNS by 12 days post infection. This system has been used as an experimental model to investigate the mechanisms of host defense against neurotropic viral infections.

Cell-mediated immune responses, particularly cytolysis by major histocompatibility complex (MHC) class I restricted $CD8^+$ T cells, play a major role in the viral clearance from infected tissues (Zinkernagel, 1993). However, the mechanism(s) of cell-mediated effect(s) in clearing viruses from infected neurons, which do not express MHC antigens, remain obscure. With the exception of sensory olfactory neurons (Margolis et al., 1991), neurons in the adult CNS can not regenerate after trauma or inflammation. Therefore, eliminating virus from neurons through destructive means is potentially detrimental to the host. Studies with neurotropic viruses such as alphavirus and rabies virus have implied the existence of a non-MHC-restricted, noncytolytic mechanisms for clearance of viral infections in neurons (Levine et al., 1991; Dietzschold et al., 1992). Our own data examining VSV infection of the CNS are consistent with the hypothesis (Plakhov et al., 1995; Huneycutt et al., 1993, 1994; Bi et al., 1995a).

NO is a physiological neurotransmitter in the CNS which can be produced constitutively by type I NOS (neuronal constitutive NOS or brain NOS) in neurons. Type III NOS (endothelial cell NOS) is also found in the CNS in both endothelial cells and a subset of neurons in

* Corresponding author. Tel.: +1-212-9988269; fax: +1-212-9954015; e-mail: carol.reiss@nyu.edu

[1] Present address: Aaron Diamond Research Institute, New York, NY, USA.

0165-5728/96/$15.00
PII S0165-5728(96)00083-5

the hippocampus; we have recently shown that CNS astrocytes synthesize type III NOS (Barna et al., 1996). Type II NOS (inducible NOS or macrophage NOS) can be found in the CNS as well, both in activated astrocytes and microglial cells in the CNS (Snyder, 1992; Corradin et al., 1993; Galea et al., 1994; Nathan and Xie, 1994). We have shown that NO inhibits VSV infection in neuroblastoma NB41A3 cells in vitro (Bi and Reiss, 1995).

Recent in vivo studies with interleukin-12, a strong activator of IFN-γ production, have suggested the importance of IFN-γ in the enhanced recovery from VSV infection in the CNS (Bi et al., 1995b; Komatsu et al., in preparation). Therefore, we initiated studies to investigate the potential role of IFN-γ in clearing neurotropic viral infections in neurons through stimulating NO production.

2. Materials and methods

2.1. Cells and viruses

NB41A3 neuroblastoma cells and C6 astrocytoma cells were purchased from ATCC. RAW murine macrophage cells were generously provided by A. Amin, NYU. Human poliovirus receptor-expressing N2A murine neuroblastoma cells were generously provided by V. Racaniello, College of Physicians and Surgeons. Vero cells were the gift of P. Schaffer, Harvard Medical School. MDBK cells were provided by P. Palese, Mt. Sinai School of Medicine. Chinese hamster ovary (CHO) cells were maintained as previously described (Huneycutt et al., 1994). Murine peritoneal macrophages were harvested by lavage with DMEM supplemented with 5% FBS and adherence on TC flasks. The mouse hybridoma, GR-20, a mAb antagonist to the IFN-γR, was purchased from ATCC, as were the rat mAb XMG1.2 and GL113.

VSV, Indiana serotype, San Juan strain was used for most of these studies as previously described (Huneycutt et al., 1994; Bi et al., 1995a). Polio type 1 was generously provided by V. Racaniello. Influenza A/WSN/33 was the gift of P. Palese. HSV-1 was provided by P. Schaffer. Sindbis AR339 virus was the gift of B. Levine.

2.2. Plaque assay of infectious viral titer

Infectious virus was quantified on cell monolayers. Monolayers were prepared by inoculating 20×10^4 cells in 1 ml per well (24 well plate, Nunc) and incubated for 2 days at 37°C. The medium was removed, 0.1 ml of each dilution of samples (10-fold serial dilutions) was added to each well, and the wells were then incubated for 30 min at 37°C. The medium was removed, 1 ml of the mixture of equal volumes of 1.8% agar (kept at 45°C) and 2 × culture medium (kept at 37°C) was added to each well, and the wells were then incubated at 37°C for 24 h. Plaques were fixed with 10% formaldehyde for 30 min and stained with 0.5% methylene blue.

2.3. Chemicals and cytokines

In some experiments inhibitors of NOS were included. L-NMA (Sigma) and 7-nitroindazole (Calbiochem) were used at 400 μM as was the control compound, indazole (Aldrich). L- and D-argenine were purchased from Sigma and were used at 5 mM.

Recombinant mouse IFN-γ was purchased from Genzyme. Recombinant mouse IL-12 was provided by Genetics Institute. In some preliminary experiments, rat conA supernatant (Browning et al., 1990), was used as a source of IFN-γ; the validity of this assumption was tested using neutralizing mAb to IFN-γ or to its receptor.

2.4. Determination of NO concentration

The concentration of NO in culture supernatants was determined by assaying its stable end-product, NO_2^- (Bredt and Snyder, 1989). Briefly, equal volumes of experimental sample and Griess reagent (1% sulfanilamidide; 0.1% *N*-1-naphthylethylene-diamine, 5% H_3PO_4) (Sigma) were incubated at room temperature for 10 min. The reaction produces a pink color, which was quantitated at 540 nm against standards in the same buffer, using an automated plate reader (Bio-Tek, model EL 309). The data is expressed as μM.

2.5. Immunohistochemical staining of frozen brain sections and NB41A3 cells

Frozen brain sections of uninfected or infected mice from a previously extensively characterized set of experiments were warmed for 10 min at room temperature before being fixed in 10% neutral buffered formalin for 10 min as previously described (Bi et al., 1995a). NB41A3 cells were cultured in F-10 medium (12.5% horse serum and 2.5% inactivated FBS) on culture chamber slides overnight and dried for 10 min at room temperature before being fixed in 10% neutral buffered formalin for 15 min. Slides of frozen sections or NB41A3 cells were washed twice in 0.1 M Tris buffer. Abs used include anti-IFN-γ receptor mAb, GR-20 (ATCC; culture supernatant), anti-neurofilament p68 mAb (Sigma; 1:25 dilution), anti-GFAP mAb (Dako; 1:20 dilution), anti-NOS type 1 peptide (Dawson, Hopkins; 1:20), anti-NOS type II mAb (Transduction Labs; 1:20), anti-NOS type III mAb (Transduction Labs; 1:20). Endogenous peroxidase activity was blocked by incubating slides in 0.3% H_2O_2 for 20 min. Background staining was blocked by preincubating slides in 1% BSA for 45 min. Slides were incubated with primary Abs for specific antigens for one hour at room temperature, and then incubated in biotinylated secondary Ab (ABC Kit, Vector Lab), followed by avidin-biotinylated peroxidase for another 30 min. Color was developed with 3,3'-Diaminobenzidine (DAB) in 0.01% H_2O_2 for 5 min. Slides were mounted in Permount™ (Sigma). Photos were taken with an Olympus BH-2 microscope equipped with an automated camera.

2.6. Biosynthetic labelling and immunopreciptation

NB41A3 cells (5×10^5) were cultured in culture medium with or without IFN-γ for 72 h. Cells were washed with serum-free, methionine-free medium and incubated in this medium containing 50 μCi/ml ^{35}S-methionine for another 20 h. Cells were then chilled on ice for 10 min and lysed with 500 μl of lysis buffer (0.5% NP-40, 300 mM NaCl, 50 mM Tris, 100 μg/ml PMSF, 1 μg/ml leupeptin, PH 7.4) for 20 min. Cell lysate was centrifuged at 12;000 g for 2 min. Equal amounts of supernatant (150 μl) was precleared with 50% protein A-sepherose 2–3 times. Polyclonal anti-ncNOS peptide (the gift of Ted Dawson, Johns Hopkins) or the control normal serum (1:15 dilution) was added to the cell lysate for one hour at 37°C. Protein A complex was pelleted down in a microfuge for one minute, and boiled for 5 min in dissociation buffer (0.05% bromphenol blue, 0.0625 M Tris, 1% SDS, 10% glycerol, 1% 2-mercaptoethanol). Samples were then analyzed on 8% SDS-PAGE gel electrophoresis with high-range molecular weight markers (Promega). The gel was then soaked in DMSO twice for 30 min each and immersed in 22.2% 2,5-diphenyloxazole for another hour. The gel was finally soaked in water for one hour and dried under vacuum. Film exposure was on Kodak Bio-Max MR film for 5 h at −70°C. Phosphorimaging analysis of the gel was applied with Bio-Rad Model GS-250 Molecular Imager™.

2.7. Experimental infection of mice

Four groups of 10 mice were infected intranasally with 2×10^5 pfu VSV, as previously described (Bi et al., 1995a,b). Mice were injected daily with either indazole 50 mg/kg or 7NI 50 mg/kg. Half of the mice received 200 ng IL-12 (Genetics Institute). At 4 days post infection, mice were sacrificed and homogenates of CNS were prepared. The titer of VSV in the individual samples was determined by plaque assay on CHO monolayers as previously described (Huneycutt et al., 1994).

2.8. Sequence analysis

The sequence of the promoter region of human type I NOS, GenBank Locus HSU 15666, was examined for the presence of well characterized IFN-γ associated footprints. Included in the survey was GAS, GATCGATTTCCCCGAAAT (Pine, 1992); IRS, CTCCTCCCTTCTGAGG*AAACGAAAC*CAACAGCAGTCCAAG (Coccia et al., 1991); STAT core sequence, TTCCCCGAA (Ivashkiv, 1995); IRF-1/ISGF2, GAAGTGAAG, AAAACCGAAA and AAACTGAAA (Benech et al., 1992); PIE region, CAATTTCC and CTCTTTTCTA (Benech et al., 1992); IFN-γ responsive sequence, GGAAAAGCA and CAGAAAAGGA (Benech et al., 1992); ISRE, CTCACGCTTT*GGAAAGTGAAACC*TACCTACTC (Ohmori and Hamilton, 1995).

3. Results

3.1. NB41A3 cells express IFN-γ receptor and type I NOS

Because we had observed an increase in immunohistochemical staining for type I NOS in brain tissue sections following infection with VSV (unpublished observation), we examined the role of type I NOS in viral clearance and the potential involvement of IFN-γ in induction of type I NOS expression. We first observed the expression of IFN-γ receptor (IFN-γR) on olfactory bulb neurons (unpublished observation) and in vitro. NB41A3 cells reacted positively for IFN-γR by using immunocytochemical staining with an anti-IFN-γR monoclonal antibody (mAb) GR-20. In addition, these cells stained positive for the neuronal marker protein, neurofilament, with an anti-neurofilament 68 mAb and did not react with mAb for the astrocyte marker protein glial fibrillary acid protein (GFAP). Further immunocytochemical characterization of NB41A3 cells detected only type I NOS expression but not the other two isoforms of NO synthases (type II NOS and type III NOS; data not shown).

3.2. IFN-γ treatment significantly increases NB41a3 cells' production of NO

NO production by NB41A3 cells was significantly increased by IFN-γ. We examined the kinetics of induction of NO_2^- secretion into the cell culture medium, contrasting the neuroblastoma cells with a murine macrophage line, RAW, frequently used to study type II NOS. Fig. 1 shows the release of NO_2^- from each source. The macrophage line reaches plateau levels of production by 24 h of incubation with 5 ng rIFN-γ. The neuroblastoma cells, do

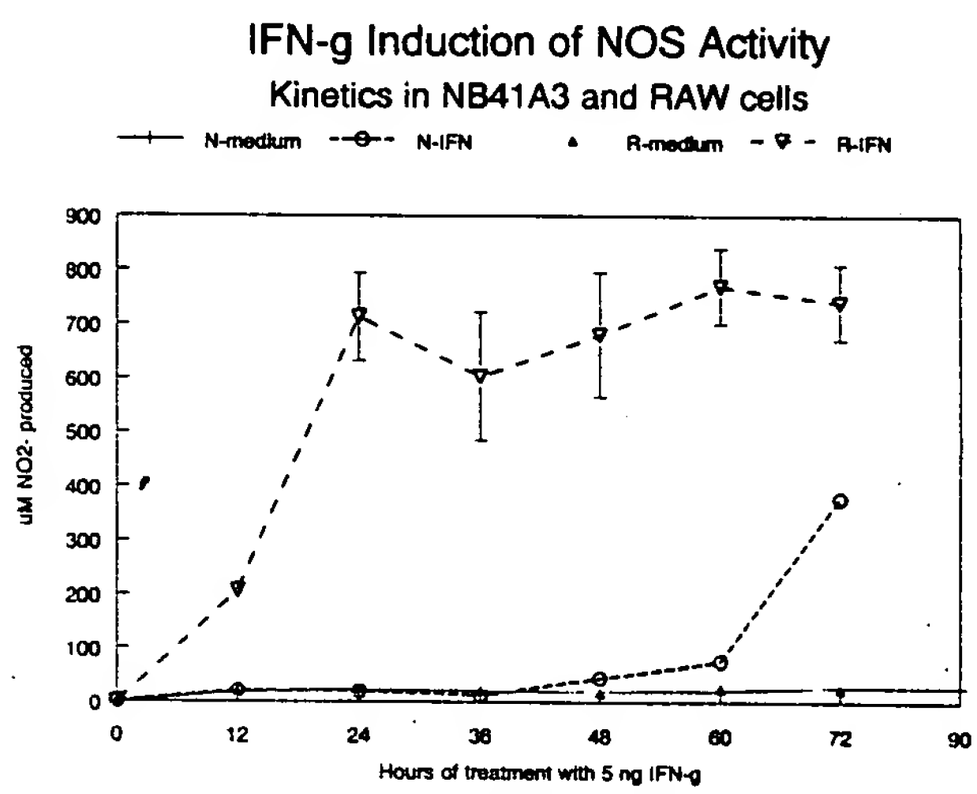


Fig. 1. Kinetics of the IFN-γ augmented NOS activity in NB41A3 and RAW cells. Aliquots of supernatant were removed from triplicate wells of 2×10^5 NB41A3 or RAW cells, cultured with medium or with 5 ng IFN-γ for up to 72 h. The medium was assayed for the presence of NO_2^- using the Greiss reagent, and expressed as nM $NO_2^- \pm$S.D. present.

not reach substantial levels of NO_2^- production until 72 h of co-culture.

Activating the neuroblastoma cells through their glutamate receptor, with 400 μM *N*-methyl-D-aspartic acid (NMDA) for two minutes, significantly increased the NO production, as well (Fig. 2). Macrophages, however, do not have the glutamate receptor, and were not activated by NMDA. The L-arginine analogue, L-*N*-methyl-arginine (L-NMA), blocked the IFN-γ stimulated NO production by NB41A3, and peritoneal macrophages (Fig. 2) and also RAW, and C6 astrocytoma cells (Table 2, discussed below), consistent with the production of NO from an L-arginine substrate by NOS. In addition, adding 5 mM L-arginine but not D-arginine efficiently reversed the inhibition of L-NMA on NO production, further confirming the substrate stereospecificity of the enzyme.

3.3. IFN-γ treatment significantly increases NB41A3 cells' expression of type I NOS

The activity of type I NOS is regulated by many factors including calcium, phosphorylation and flavin concentration, and, unlike type II NOS, it is generally accepted that type I NOS enzyme is a long-lived, constitutively expressed protein (Snyder, 1992; Nathan and Xie, 1994; Bredt and Snyder, 1994). However, constitutively expressed type I and III NOSs are still subjected to dramatic induction at the transcriptional level (Weiner et al., 1994; Verge et al., 1992; Roskams et al., 1994). We investigated whether IFN-γ regulates the synthesis of type I NOS. Immunoprecipitation of biosynthetically labelled type I NOS protein from IFN-γ treated NB41A3 cells followed by SDS-PAGE and autoradiography revealed that the amount of type I NOS protein was increased in a dose-dependent fashion (Fig. 3). Phosphorimager analysis of the gel indicated that IFN-γ treatment increased the amount of type I NOS protein by 200%.

3.4. IFN-γ induced upregulation of type I NOS activity inhibits VSV replication

We then investigated whether viral replication in NB41A3 cells could be inhibited by IFN-γ-induced type I NOS. Treatment of NB41A3 cells for 72 h prior to infection significantly inhibited VSV and HSV-1 replication (Table 1). Replication of Influenza virus A/WSN/33 and Sindbis virus in NB41A3 cells was also significantly inhibited. In other experiments, the IFN-γ mediated reduction in viral propagation was prevented by addition of anti-IFN-γR mAb GR-20, not shown. The abrogation of IFN-γ-induced inhibition of VSV and HSV-1 replication by the L-arginine analogues L-NMA and 7NI suggest that the IFN-γ-induced inhibition is due to type I NOS activity. In contrast, while IFN-γ treatment inhibited influenza and Sindbis virus replication, this was not reversible with argenine analogs. The data suggest that influenza is susceptible to other IFN-γ-induced anti-viral enzymes (Staeheli, 1990), but not to NO-mediated inhibition.

Treating NB41A3 cells with 500 μM NMDA for two minutes also significantly inhibited VSV replication; anti-IFN-γR, GR-20 did not reverse the NMDA-induced inhibition of VSV replication in NB41A3 cells (not shown). Influenza virus replication, in contrast, and was not altered by NMDA-stimulation of the neurons. This is consistent with the insensitivity to NO-mediated inhibition of influenza virus replication.

3.5. NO production in neurons also inhibits polio virus replication

We also investigated the susceptibility of polio virus to NO-mediated inhibition in vitro. NO donor *S*-nitro-*N*-acetyl-penicillamine (SNAP) significantly inhibited type I polio virus replication in human polio-virus-receptor-transfected mouse neuroblastoma N2A cells (Fig. 4). Treatment with control compound, *N*-acetyl-penicillamine (NAP), which is not a nitrogen donor for NOS activity, had no effect on either viral replication.

3.6. Inhibition of VSV replication in NB41A3, but not RAW and C6 cells is attributable to type 1 NOS activity

7-Nitroindazole (7NI) is a selective inhibitor of type 1, but not types II or III NOS (Moore et al., 1993). Therefore, we incubated cells expressing the 3 isoforms of NOS with IFN-γ, NMDA, or medium and 2 inhibitors. L-NMA and

Table 1
Interferon-γ-induced viral inhibition in NB41A3 cells: growth of VSV and HSV-1 is sensitive to nitric oxide-mediated inactivation, but Influenza and Sindbis viruses are resistant to NOS-inhibition

Inhibitor [a]	VSV		Influenza		Sindbis		HSV-1	
	media	IFN-γ	media	IFN-γ	media	IFN-γ	media	IFN-γ
Media	5.615 ± 0.282 [b]	*3.752 ± 0.034* [c]	5.683 ± 0.140	*3.810 ± 0.130*	4.859 ± 0.089	*3.318 ± 0.373*	5.016 ± 0.176	*3.467 ± 0.408*
7NI	5.985 ± 0.428	5.752 ± 0.331	5.560 ± 0.489	*3.897 ± 0.089*	4.935 ± 0.056	*3.897 ± 0.089*	5.159 ± 0.194	5.170 ± 0.264
Indazole	5.460 ± 0.408	*3.678 ± 0.174*	5.268 ± 0.350	*3.546 ± 0.212*	5.053 ± 0.217	*3.752 ± 0.046*	5.140 ± 0.204	*3.175 ± 0.369*
L-NMA	5.948 ± 0.089	5.761 ± 0.151	5.810 ± 0.131	*3.767 ± 0.208*	5.033 ± 0.238	*3.796 ± 0.084*	5.359 ± 0.225	5.227 ± 0.208

[a] Cultures of NB41A3 cells were stimulated with media or 5 ng IFN-γ for 72 h, prior to 8 h infection with VSV, A/WSN/33, Sindbis AR339, or HSV-1 at a moi = 1; in some cultures NOS inhibitors 7NI and L-NMA were added at 400 μM. Supernatants were assayed for infectious virus on NB41A3 monolayers.

[b] Data is expressed as $\log_{10}$ pfu $\pm$ S.D.

[c] Italic data is significantly different from control values: $p < 0.001$.

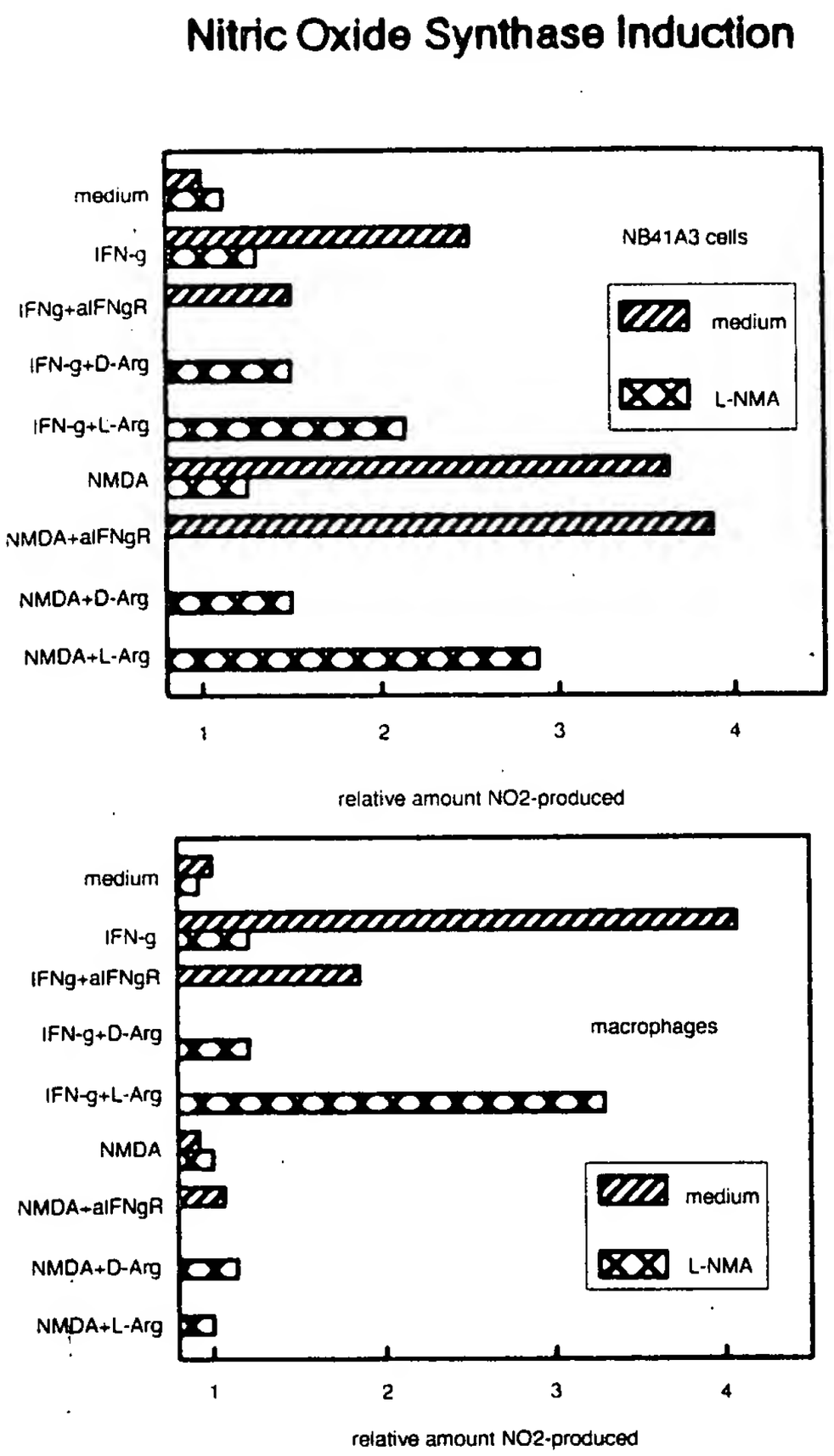


Fig. 2. Nitric oxide synthase induction of NB41A3 cells and macrophages. 2×10^5 NB41A3 cells or peritoneal macrophages were cultured for 72 h with medium or with a crude source of IFN-γ, rat conA supernatant. Replicate wells were cultured with medium (hatched bar) or with the inhibitory argenine analog L-NMA (500 μM; crosshatched bar). Some wells were incubated as well with GR-20, a mAb to the IFN-γ receptor which antagonizes IFN-γ action, with 5 mM D-argenine or L-argenine. Other sets of wells were cultured for 2 min with 500 μM NMDA to activate neurons through their glutamate receptor. Nitrite production was determined by the Greiss assay. Maximal and minimal production for NB41A3 cells were 31 and 8 μM, respectively; for macrophages the responses were 57 and 14. The relative change from background was determined.

7NI. The cells were infected with VSV and the progeny virus was determined 8 h later by plaque assay. L-NMA antagonized NOS-associated inhibition of viral replication in all three cell lines, whether NOS was activated by triggering of the cells through their glutamate receptors, or by IFN-γ treatment (Table 2). 7NI treatment was controlled with indazole incubation. Only neuronal NOS was antagonized with 7NI, the resultant virus produced in

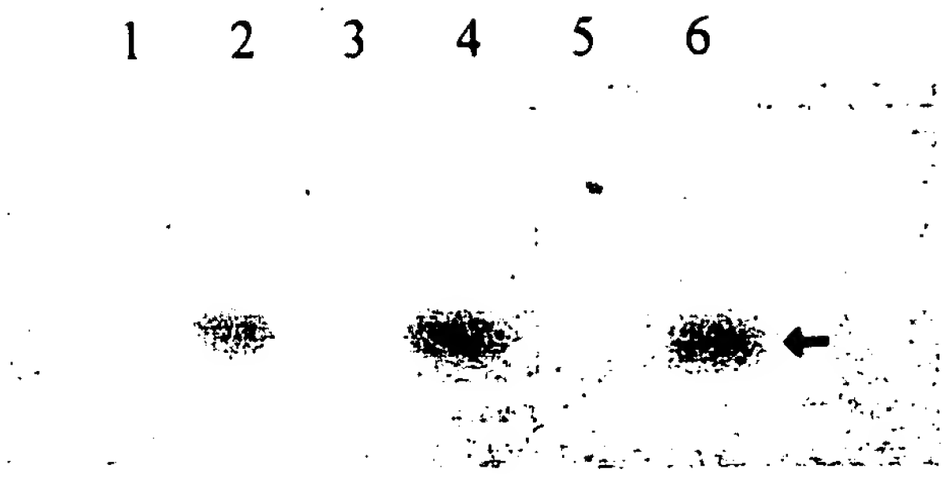


Fig. 3. IFN-γ increases type I NOS synthesis in NB41A3 cells. 5×10^5 NB41A3 cells were cultured in either medium (lanes 1, 2), 1 ng IFN-γ (lanes 3, 4) or 10 ng IFN-γ (lanes 5, 6) for 72 h. Cells were than biosynthetically labelled with 50 μCi/ml ^{35}S-methionine for 20 h. An equal amount of cell lysate of each condition (150 μl) was then immunoprecipitated with 1:15 dilution of either the polyclonal anti-type I NOS peptide Ab (lanes 2, 4, 6) or control normal serum (lanes 1, 3, 5). Arrow indicates the band is at 155 kD. Phosphorimager analysis of the radioactive intensity of each band determined that lanes 4 and 6 were 1.2 fold and 2.1 fold (respectively) the incorporation of ^{35}S-methionine into type I NOS when compared with cells treated with medium.

RAW and C6 cells was indistinguishable from medium- or from indazole-treated activated cells.

3.7. 7NI treatment of mice alters the course of viral replication in the CNS

To determine whether type I NOS activity was biologically relevant in the CNS of mice infected intranasally, groups of BALB/c male mice were infected with VSV and were injected with either indazole or with 7NI. In addition, half of mice were injected with IL-12, which we have previously shown has profound recovery-promoting effect(s) in this experimental system. Four days later, the

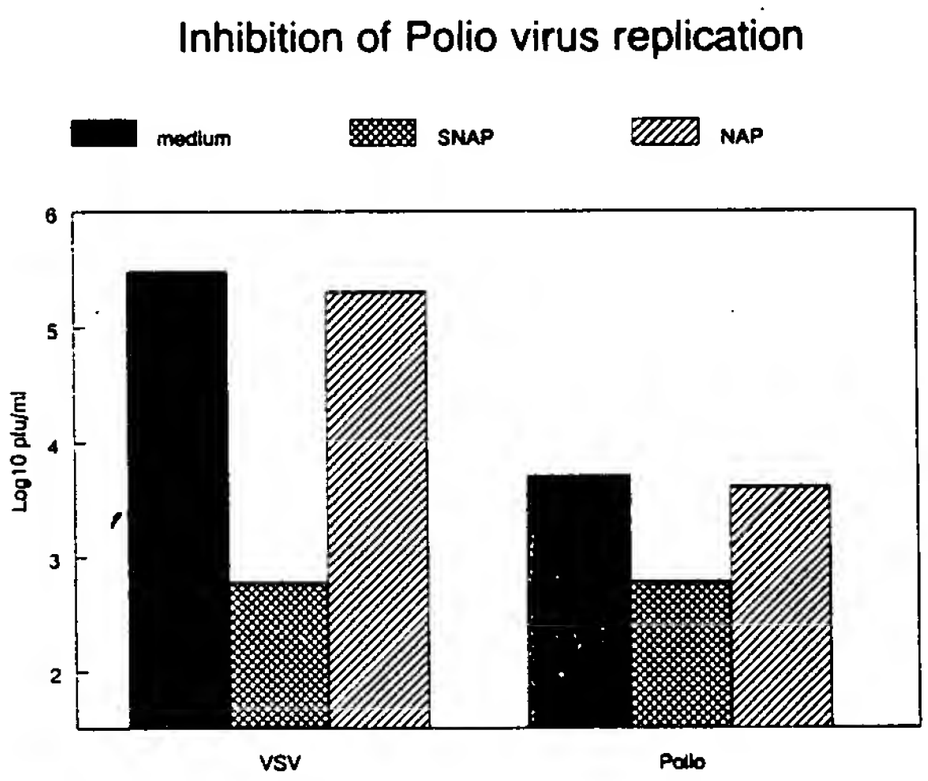


Fig. 4. Poliovirus replication is inhibited by NO. Type 1 polio virus and VSV were used to infect N2A cells expressing the human polio virus receptor, moi = 1, in the presence or absence 100 μM SNAP, a nitrate donor, added every 4 h, as previously described (Bi and Reiss, 1995). After overnight incubation, supernatant virus progeny was titrated by plaque assay. Data shown are representative of two replicate assays.

Table 2
VSV infection in the presence of NOS inhibitors

Cells	Inhibitor [a]	Medium	NMDA	IFN-γ
RAW mϕ	medium	5.79 ± 0.09	–	*4.55 ± 0.49* [b]
RAW mϕ	7NI	5.87 ± 0.08	–	*4.84 ± 0.15*
RAW mϕ	L-NMA	5.83 ± 0.13	–	5.71 ± 0.093
RAW mϕ	indazole	5.45 ± 0.21	–	*4.49 ± 0.48*
NB41A3 neuroblastoma	medium	5.80 ± 0.10	*3.71 ± 0.20*	*3.94 ± 0.063*
NB41A3	7NI	5.76 ± 0.18	5.57 ± 0.15	5.76 ± 0.097
NB41A3	L-NMA	5.76 ± 0.25	5.59 ± 0.27	5.77 ± 0.21
NB41A3	indazole	5.58 ± 0.27	*4.39 ± 0.36*	*4.23 ± 0.29*
C6 astrocyte	medium	5.89 ± 0.21	*4.54 ± 0.24*	*4.41 ± 0.37*
C6	7NI	5.83 ± 0.14	*4.59 ± 0.52*	*4.54 ± 0.14*
C6	L-NMA	5.85 ± 0.08	5.38 ± 0.37	5.59 ± 0.26
C6	indazole	5.63 ± 0.19	*4.48 ± 0.17*	*4.48 ± 0.42*

[a] In vitro conditions: Inhibitors were used at 400 μM. NMDA at 500 μM for 2 min. IFN-γ at 5 ng for 72 h. initial infection moi = 1. data shown is $\log_{10}$ pfu virus ± S.D. derived from supernatants harvested 8 h pi.
[b] Italic data points are significantly different from uninhibited viral replication. $p < 0.05$ or better.

mice were sacrificed, and brain homogenates were tested for the presence of virus. Fig. 5 shows the results of the plaque assay on homogenates. The geometric mean titer (GMT) of virus in individuals within each group was compared. 7NI treatment of mice resulted in a 10-fold greater GMT compared to indazole treated mice. In addition, 7NI treatment abrogated the IL-12-mediated enhanced clearance of VSV. This is consistent with our hypothesis that IL-12 induced IFN-γ which in turn stimulated NOS in the CNS. These data clearly demonstrate the substantial contribution of type I NOS to restricting viral replication within neurons of the CNS during experimental VSV infection.

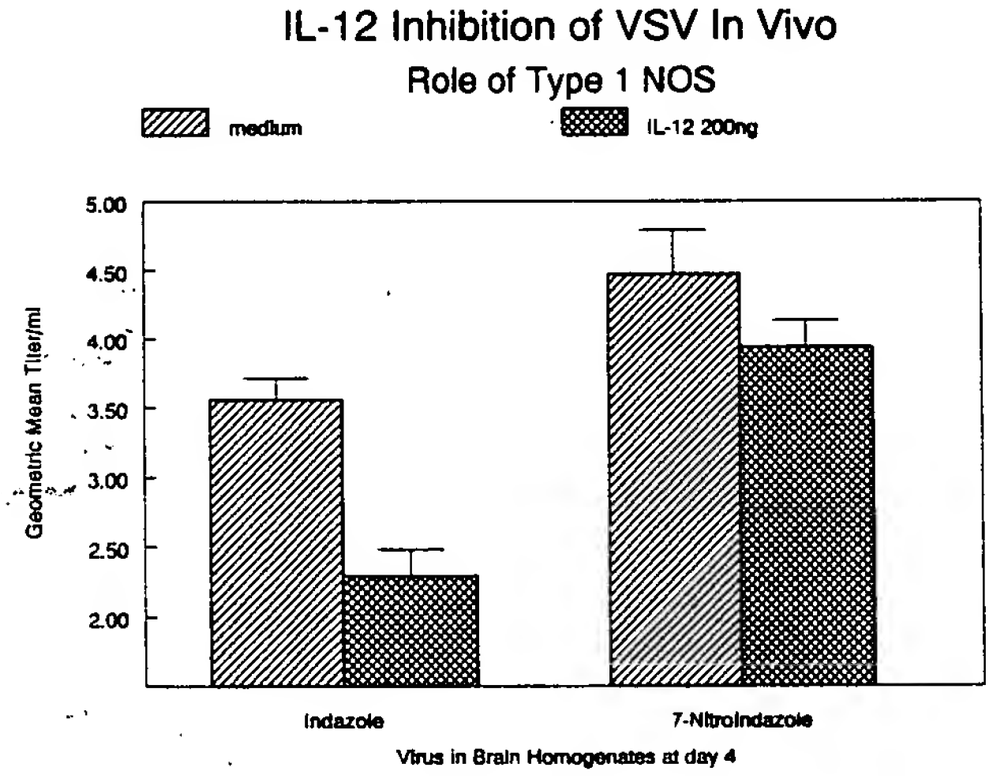


Fig. 5. IL-12 inhibition of VSV in vivo: role of type 1 NOS. Four groups of 10 mice each were injected with 50 mg/kg indazole or with 7-nitroindazole and medium (hatched bars) or with 200 ng IL-12 (cross-hatched bars) and were infected intranasally with 2×10^5 pfu VSV. At 4 days post infection, mice were sacrificed and brain homogenates were prepared. The amount of virus in individual samples was determined by plaque assay on CHO monolayers. Geometric mean titers ± SEM are shown.

4. Discussion

These studies demonstrate that IFN-γ can inhibit VSV replication through induction of the synthesis and activity of type I NOS in neurons in vitro (Tables 1 and 2) and in vivo (Fig. 5). We have shown that this antiviral effect in culture is not limited to VSV, but can be extended to poliovirus type 1 and HSV-1 (Table 1, Fig. 4). Little is known about the mechanism(s) of IFN-γ regulation of type I NOS at present. It is possible that IFN-γ increases NOS gene expression at the transcription level, or IFN-γ increases the quantity of NOS posttranscriptionally by either increasing the half-life of NOS mRNA or stabilizing the NOS protein. IFN regulatory factor (IRF)-1 is required in iNOS induction in mouse macrophage (Kamijo et al., 1994). IFN-γ signal transduction in neurons, however, may or may not be similar to that in other types of cells. Two closely linked, but separable, promoters of human type I NOS have been identified (Xie et al., 1995). Our analysis of the sequence of the promoter region of human type I NOS has suggested a STAT core element and possible sites for PIE and GAS. IRS, IRF-1, IFN-γ responsive sequence and interferon stimulation responsive elements were not found. However, human type II NOS gene behaves differently than the mouse gene, and is not readily inducible by IFN-γ, TNF-α or LPS (Reiling et al., 1994). There may be other cytokine response elements in the 5′ region of the gene, and only future investigations, and isolation of the murine promoter(s) will provide the insights necessary.

It has been previously demonstrated that IFN-γ can inhibit several viral infections in macrophages through iNOS induction (Karupiah et al., 1993). This is the first report that IFN-γ can inhibit VSV in neurons through inducing type I NOS (Table 2). NO inhibits replication of polio virus and HSV-1 in neurons (Table 1, Fig. 4). Considering that NO-generating neurons are selectively resistant to neurotoxicity of NO (Dawson et al., 1991), one more advantage can be attributed to IFN-γ-mediated activation of NOS in neurons in inhibiting viral infections in the CNS rather than simply just induction of iNOS in neighboring neuroglial cells.

Alternative noncytolytic means of clearing viral infections in neurons such as antibody-mediated clearance have been demonstrated in other neurotropic viral infections (Levine et al., 1991; Dietzschold et al., 1992). But since neither antibodies to VSV nor B cells infiltrating the CNS are observed before day 10, this mechanism is unlikely to be essential in clearance of VSV infection in the CNS in immunocompetent mice (Bi et al., 1995a). Acute viral infection of neurons should be rapidly controlled by the

host. While other anti-viral factors may exist, type I NOS may the most important anti-viral factor of the host innate immunity existing in neurons.

Acknowledgements

This work was supported by a grant from the NIH AI18083 and a Bridge grant from the Faculty of Arts and Science of NYU to CSR. Ted Dawson, Johns Hopkins University generously provided anti-NOS type I peptide antibody and provocative discussions. Other discussions of the work in progress which were stimulating were with David Bredt (University of California, San Francisco), Anthony Young (Ohio State), FY Liew (Glasgow), and Alice Huang (NYU). Viruses and cell lines were generously provided by V. Racaniello and B. Levine (College of Physicians and Surgeons), P. Schaffer (Dana-Farber Cancer Institute), and P. Palese (Mt Sinai School of Medicine). IL-12 was generously provided by Genetics Institute. We would like to thank Dr. Shirley Bartido for assistance with the immunoprecipitation, electrophoresis, and autoradiography, and Shelli Ann Oien for technical support.

References

Barna, M., Komatsu, T. and Reiss, C.S. (1996) Activation of type III nitric oxide synthase in astrocytes following a neurotropic viral infection. Virology, in press.

Benech, P.D., Sastry, K., Iyer, R.R., Eichbaum, Q., Ravech, D.P. and Ezekowitz, R.A.B. (1992) Definition of interferon-γ-response elements in a novel human Fcγ receptor gene (FcγRIb) and characterization of the gene structure. J. Exp. Med. 176, 1115–1123.

Bi, Z., Barna, M., Komatsu, T. and Reiss, C.S. (1995a) Vesicular stomatitis virus infection of the central nervous system activates both innate and acquired immunity. J. Virol. 69, 6466–6472.

Bi, Z., Quandt, P., Komatsu, T., Barna, M. and Reiss, C.S. (1995b) IL-12 promotes enhanced recovery from vesicular stomatitis virus infection of the central nervous system. J. Immunol. 155, 5684–5689.

Bi, Z. and Reiss, C.S. (1995) Inhibition of vesicular stomatitis virus infection by nitric oxide. J. Virol. 69, 2208-2213.

Bredt, D.S. and Snyder, S.H. (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc. Natl. Acad. Sci. USA 86, 9030–9033.

Bredt, D.S. and Snyder, S.H. (1994) Nitric oxide: a physiological messenger molecule. Ann. Rev. Biochem. 63, 175–195.

Browning, M.J., Huang, A.S. and Reiss, C.S. (1990) Cytolytic T lymphocytes from the BALB/c-H-2^{dm2} mouse recognize the vesicular stomatitis virus glycoprotein and are restricted by Class II MHC antigens. J. Immunol. 145, 985–994.

Coccia, E.M., Vaiman, D., Raber, J., Marziali, G., Fiorucci, G., Orsatti, R., Cohen, B., Nissim, N., Romeo, G., Affabris, E., Chebath, J. and Battistini, A. (1991) Protein binding to the interferon response enhancer correlates with interferon induction of 2′,5′-oligoadenylate synthetase in normal and interferon-resistant friend cells. J. Virol. 65, 2081–87.

Corradin, S.B., Mauel, J., Donini, S.D., Quattrocchi, E. and Ricciardi-Castagnoli, P. (1993) Inducible nitric oxide synthase activity of cloned murine microglial cells. Glia 7, 255–262.

Dawson, V.L., Dawson, T.M., London, E.D., Bredt, D.S. and Snyder, S.H. (1991) Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. Proc. Natl. Acad. Sci. USA 88, 6368–6371.

Dietzschold, B., Kao, M., Zheng, Y.M., Chen, Z.Y., Maul, G., Fu, Z.F., Ruprecht, C.E. and Koprowski, H. (1992) Delineation of putative mechanisms involved in antibody-mediated clearance of rabies virus from the central nervous system. Proc. Natl. Acad. Sci. USA 89, 7252–7255.

Forger, J.M., Bronson, R.T., Huang, A.S. and Reiss, C.S. (1991) Murine Infection by vesicular stomatitis virus: initial characterization of the H-2^d system. J. Virol. 65, 4950–4958.

Galea, E., Reis, D.J. and Feinstein, D.C. (1994). Cloning and expression of inducible nitric oxide synthase from rat astrocytes. J. Neurosci. Res. 37, 406–414.

Huneycutt, B.S., Bi, Z., Aoki, C. and Reiss, C.S. (1993) Central neuropathogenesis of vesicular stomatitis virus infection of immunodeficient mice. J. Virol. 67, 4980–4987.

Huneycutt, B.S., Plakhov, I.V., Shusterman, Z., Bartido, S.M., Huang, A.S., Reiss, C.S. and Aoki, C. (1994). Distribution of vesicular stomatitis virus proteins in the brain of BALB/c mice following intranasal inoculation: an immunohistochemical analysis. Brain Res. 635, 81–95.

Ivashkiv, L.B. (1995) Cytokines and STATs: How can signals achieve specificity? Immunity 3, 1–4.

Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., Shapiro, D., Lee, J., Koh, S.I., Kimura, Y, Green, S.J., Mak, T.W., Taniguchi, T. and Vilcek, J. (1994) Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. Science 263, 1612–1615.

Karupiah, G., Xie, Q.W., Buller, R.M.L., Nathan, C., Duarte, C. and Macmicking, J.D. (1993) Inhibition of viral replication by interferon-γ induced nitric oxide synthase. Science 261, 1445–1448.

Levine, B., Hadwick, J., Trapp, B., Crawford, T., Bollinger, R. and Griffin, D. (1991) Antibody-mediated clearance of alphavirus infection from neurons. Science 254, 856–860.

Lundh, B., Kristensson, K. and Norrby, E. (1987) Selective infection of olfactory and respiratory epithelium by vesicular stomatitis and Sendai viruses. Neuropathol. Appl. Neurobiol. 13, 111–122.

Margolis, F.M., Verhaagen, J., Biffo, S., Huang, F.L. and Grillo, M. (1991) Progress in Brain Research. Elsevier Science Publishers, pp. 97–120.

Moore, P.K., Wallace, P., Gaffen, Z., Hart, S.L. and Babbedge, R.L. (1993) Characterization of a novel nitric oxide synthase inhibitor 7-Nitroindazole and related indazoles: anti-nociceptive and cardiovascular effects. Br. J. Pharmacol. 110, 219–224.

Nathan, C. and Xie, Q.W. (1994) Nitric oxide synthase: roles, tolls and controls. Cell 78, 915–918.

Ohmori, Y. and Hamilton, T.A. (1995) The interferon-stimulated response element and a κB site mediate synergistic induction of murine IP-10 gene transcription by IFN-γ and TNF-α. J. Immunol. 154, 5235–5244.

Pine, R. (1992). Constitutive expression of an ISGF2/IRF1 transgene leads to interferon-independent activation of interferon-inducible genes and resistance to virus infection. J. Virol. 66, 4470–4478.

Plakhov, I.V., Arlund, E.E., Aoki, C. and Reiss, C.S. (1995) The earliest events in VSV infection of the murine olfactory neuroepithelium and entry of the central neurons in vivo. Virology 209, 257–262.

Reiling, N., Ulmer, A.J., Duchrow, M., Ernst, M., Flad, H. and Hauschildt, S. (1994) Nitric oxide synthase: mRNA expression of different isoforms in human monocytes/macrophages. Eur. J. Immunol. 24, 1941–(1945).

Roskams, A.J., Bredt, D.S., Dawson, T.M., Ronnett, G.V. (1994) Nitric oxide mediates the formation of synaptic connections in developing and regenerating olfactory receptor neurons. Neurone 13, 289–299.

Snyder, S.H. (1992) Nitric oxide: first in a new class of neurotransmitter? Science 257, 494–496.

Staeheli, P. (1990). Interferon-induced proteins and the anti-viral state. Adv. Virus Res. 38, 147–200.

Verge, V.M.K., Xu, Z., Xu, X.J., Wiesenfeld-Hallin, Z. and Hokfelt, T. (1992) Marked increase in nitric oxide synthase mRNA in rat dorsal root ganglia after peripheral axotomy: in situ hybridization and functional studies. Proc. Natl. Acad. Sci. USA 89, pp. 11617–11620.

Weiner, C.P., Lizasoain, I., Baylis, S.A., Knowles, R.G., Charles, I.G. and Moncada, S. (1994) Induction of calcium-dependent nitric oxide synthase by sex hormones. Proc. Natl. Acad. Sci. USA 91, pp. 5212–5216.

Xie, J., Roddy, P., Rife, T.K., Murad, F. and Young, A.P. (1995) Two closely linked but separable promoters for human neuronal nitric oxide synthase gene transcription. Proc. Natl. Acad. Sci. USA 92, pp. 1242–1246.

Zinkernagel, R.M. (1993) Viral immunology, 3rd Ed. In: ed. W.E. Paul, Fundamental Immunology. Raven Press, New York, pp. 1211–1250.